THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XV JUNE, 1934 No. 3

THE OXYTOCIC PROPERTY OF THE BLOOD AND ITS RELATION TO MILK FEVER IN COWS.

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Received for publication April 4th, 1934.

IN a previous paper (Bell and Morris, 1934) the authors have dealt with the oxytocic property of the blood of the cow. The blood was deproteinized by acidifying and boiling, and the neutralized extract was tested for oxytocic power by means of the excised guinea-pig uterus method (Burn and Dale, 1922), using as standard a dilution of " pituitrin " (Parke Davis & Co.).

The results briefly were as follows: With a few exceptions the oxytocic power of cow's blood is low; during the last week of pregnancy the oxytocic power increases (average of 5 oxytocic units per litre). Immediately after parturition the oxytocic power falls to a low value. The oxytocic substance of blood is more labile than pituitrin. It is not histamine or calcium. Its amount is increased for a short time by the intramuscular injection of pituitrin.

During the course of the above investigation a number of blood samples were taken from cows showing typical signs of milk fever. In this paper the results of determinations on these samples are recorded and discussed.

RESULTS.

a) *Controls.*

All the cases of milk fever occurred during the first week of the puerperium. We have taken as controls six animals which showed no abnormal symptoms in the puerperium. None of the samples showed any measurable oxytocic value, *i. e.* the values were in every instance less than 1·0 o.u. (oxytocic units) per litre. A typical result is shown in Fig. 1.

(b) *Milk Fever Cases.*

Case 1.—Seventeen hours post-partum. Sixth calf. Cow staggering, but not comatose. Blood, taken before inflation of the udder, contained 18 o.u. per litre (Fig. 2).

Case 2.—Three days post-partum. Lying on side, unable to rise, but not comatose. Symptoms suggestive of milk fever had been noted 12 hours previously. Mammary blood taken before inflation contained 2 o.u. per litre.

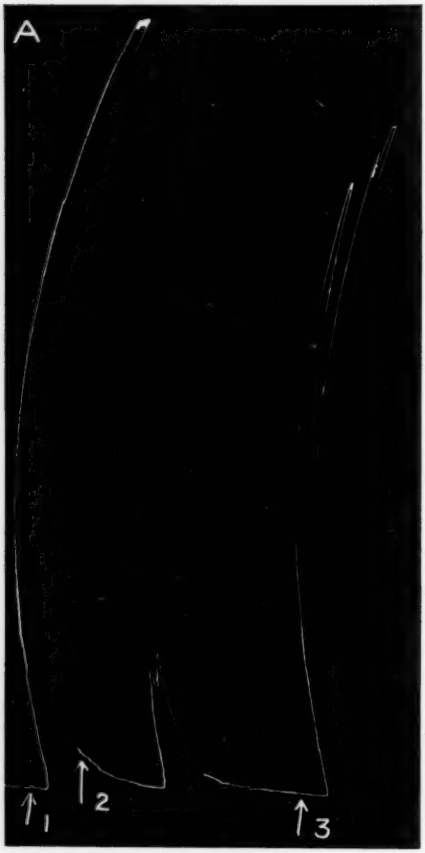

FIG. 1.

FIG. 2.

T denotes the blood extract made in the manner indicated in the first paragraph of the introduction. S denotes the dilution of " pituitrin " containing 20 oxytocic units per litre.
FIG. 1.—Control case 24 hours after calving: (1) 0·2 c.c. S; (2) 2·5 c.c. T; (3) 0·1 c.c. S.
FIG. 2.—Milk-fever case: (1) 1·0 c.c. S; (2) 1·3 c.c. T; (3) 1·0 c.c. S; (4) 1·1 c.c. T.

Case 3.—Ten hours post-partum. Stiff and staggering, but not lying down. Jugular blood taken before inflation contained 3·3 o.u. per litre.

Case 4.—Sixteen hours post-partum. Lying on side, very stiff, but not

comatose. Stood up after inflation of the udder. Jugular blood taken one hour after inflation contained 3 o.u. per litre.

Case 5.—Udder inflated 18 hours post-partum. Recurrence three days later. Cow was staggering about, muscles were very stiff, no coma. Jugular blood taken before inflation contained 2 o.u. per litre.

Case 6.—Nothing abnormal noted during the pregnancy, fifth calf. After the calving it was suspected that there was some eversion of the uterus. Two hours later she lay on her side rigid, but not comatose. The udder was inflated after taking a blood sample. She was sick and did not eat well for several days.

Three days ante-partum . . .	Less than 1·0 o.u. per litre.
Two hours ,, . . .	2·7 ,, ,,
,, ,, post-partum . . .	3·5 ,, ,,
Four hours ,, . . .	5·0 ,, ,,
Seventeen hours post-partum . .	2·5 ,, ,,
Sixty-five hours ,, . .	Less than 1·0 ,, ,,

Briefly these results show that the oxytocic power of the blood in milk-fever cases is about the same as that which is normally found in the last week of pregnancy. There is insufficient information to relate the oxytocic content to the severity of the symptoms.

DISCUSSION.

Milk fever occurs in well-nourished cows usually 10 to 72 hours after calving. The animals are nearly always noted as high milk yielders. It is often observed at the time of calving that the udder is less distended than normal. When the condition has developed, the cow trembles and staggers, and may fall down ; the leg muscles become very rigid, she pays no attention to needle jags. Coma and death supervene if the case is untreated.

Before 1896 the theories as to the causation of milk fever were speculative and of historical interest only. In that year Schmidt injected KI solution into the udder, with immediate success. Later it was found that injection of water, or even of air, was sufficient to bring about a cure of the condition ; the latter is now the universal method of treatment.

In 1925 Little and Wright found that cases of milk fever showed a low serum Ca ; this was corroborated by Dryerre and Greig (1928). Both authors later reported that intravenous injection of calcium salts cured the condition. In 1925 Widmark and Carlens claimed that milk fever was due to a hypoglycæmia ; it has since been shown that there is, if anything, a slight increase in the blood-sugar (Hayden and Fish, 1927). Fish (1929) has also shown that the inorganic phosphate of the blood is lowered in cases of milk fever.

As already mentioned, there is an increase of the oxytocic property of the blood after intramuscular injection of pituitrin. In view of the increase of the oxytocic property which occurs in milk fever, it is of interest to compare the biochemical results following injection of pituitrin with the biochemical findings summarized in the previous paragraph.

It has long been known that injection of posterior pituitary substance raises the blood-sugar. It has been shown by Nitzescu and Benetato (1930) that pitocin and pitressin produce, in rabbits, an increase in inorganic phosphate as well as a hyperglycæmia. Urechia, Groze and Retzeneau (1930) state that there is a lowering of blood Ca in man an hour after the intravenous injection of pitressin, with a small increase or decrease in inorganic phosphate. According to these authors pitocin has very little effect on blood Ca, but tends slightly to increase inorganic phosphate.

It is a common practice to starve for 24 hours before delivery an animal which is likely to develop milk fever. Starvation has been shown (Maclean, 1928) to reduce the oxytocic content of the blood.

All these considerations suggest that pituitary hormone may play a part in the causation of milk fever, the excess of this hormone in the blood at a time of hormonal adjustment and imbalance producing the changes which are responsible for the symptoms of milk fever.

If this suggestion is correct, it might be expected that injection of pituitrin into a recently calved animal would produce the symptoms of milk fever. The high cost of experiments which involve the use of cattle has prevented us from investigating this point. Moreover, one cannot substitute smaller animals in such experiments, since milk fever is a disease of very rare occurrence in animals other than the cow. We have, however, one case to report. The result is unconvincing.

This cow had had milk fever at three previous calvings. As she showed no signs of the condition 29 hours post-partum a blood sample was taken, and immediately thereafter 20 c.c. of pituitrin were injected intramuscularly. The blood sample was found to contain 15 o.u. per litre. Two hours after the injection another blood sample was taken, and this contained less than 0·6 o.u. per litre. Not only, therefore, was there no recovery of the injected pituitrin, but the original oxytocic power of the blood had itself vanished. On the next morning (*i. e.* two days post-partum) a further blood sample was taken, and immediately afterwards 10 c.c. of pituitrin were injected intramuscularly. The blood sample showed 10 o.u. per litre. A sample taken $2\frac{3}{4}$ hours later showed 4 o.u. per litre. Thus, there was again a fall in the oxytocic power of the blood following injection of pituitrin.

This cow did not at any time show signs of milk fever. Unfortunately she went out of our observation after the second day.

The above results can be interpreted in two ways: First, that pituitrin injections will not produce milk fever, and that excess pituitrin secretion is not the primary cause of this condition; or second, that we administered an inappropriate dose at an inappropriate moment. Morris (1933) has shown that the effect of pituitrin on metabolism varies with the dosage. Thus, he found that whereas small doses of pituitrin injected subcutaneously caused an antidiuresis and a decrease in nitrogen excretion, large doses gave a diuresis and a large excretion of urinary nitrogen. His results indicate, therefore, that above a certain minimum the action of pituitrin may be reversed. It seems possible that our failure to induce milk fever symptoms by pituitrin administration may have been due to excessive dosage.

SUMMARY.

1. Six cases of milk fever in cows are reported, in which the oxytocic content of the blood was found to average 3 o.u. per litre. Six controls showed no measurable oxytocic content.

2. The biochemical findings in milk fever are compared with those occurring after injection of pituitrin. These suggest that milk fever may be due to excessive or continued secretion of pituitary hormone after parturition.

3. An unsuccessful attempt to confirm this experimentally is described.

We wish to express our indebtedness to Mr. W. A. Macgregor, M.R.C.V.S., D.V.S.M., and Mr. David Weir, M.R.C.V.S., who secured the blood samples for us, and to Mr. A. B. Fowler, B.Sc., who gave us great assistance with animals on the Institute farm. We wish to thank Dr. N. C. Wright for his continued interest in this work.

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THE RELATION OF THE ELECTRIC CHARGE OF THE RED CELLS TO PHAGOCYTOSIS IN AVIAN MALARIA.

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Received for publication April 10th, 1934.

DURING the past few years considerable attention has been paid to the mechanism by which immunity, or more correctly, tolerance, latency or premunition, is acquired in protozoal infections. Avian malaria in particular has been the subject of many investigations.

When canaries are inoculated with an avian plasmodium there follows, after an interval of some days, a rapid increase in the number of parasites in the peripheral blood-stream, the length of the incubation period depending on the strain of parasite and the number of infected red cells injected. When a high percentage of the hosts' red cells has become infected, either death takes place, or a dramatic recovery associated with the rapid disappearance of infected red cells from the peripheral blood-stream occurs. In birds which have recovered and have become tolerant infection persists for life, and occasional relapses may occur, either spontaneously, or as a result of experimental procedures such as the administration of adrenalin or exposure to X-rays.

The mechanism by which infected red blood-corpuscles are removed from the peripheral blood-stream has been studied by W. H. and L. G. Taliaferro (1929), Cannon and Taliaferro (1931) and Torrioli (1932). The findings obtained by these workers may be summarized as follows. During an acute primary infection of canaries, phagocytosis of malaria parasites at all stages of their development occurs, beginning as early as four hours after inoculation. At the time of crisis there is evidence of increased phagocytic activity and proliferation of reticulo-endothelial cells, especially in the liver and spleen.

After tolerance has been acquired, canaries injected intravenously with a large dose of parasites of the homologous strain continue to exhibit active phagocytosis by the reticulo-endothelial cells of the liver and spleen.

SEROLOGICAL CHANGES IN AVIAN MALARIA.

The functional activity of the reticulo-endothelial system is thus of paramount importance in removing infected red cells from the peripheral blood of the canary; the question, however, remains as to the possible *rôle* of the serum in promoting this activity.

So far no very satisfactory proof of any serological mechanism has been brought forward to account for the tolerance in avian malaria. Lotze (1932) claimed that parasites may be made to disappear from the circulation by the intramuscular injection of freshly drawn blood or serum from an infected

bird, or by the inoculation of a saline suspension of parasitized blood-corpuscles. Moldovan (1912) and Taliaferro and Taliaferro (1929), however, were unable to show that the serum of birds with latent infections had any protective power or sensitizing action on infected red cells. Gingrich (1932) suggested that in the acquirement of tolerance there are two reactions, one, the more important, a specific mechanism, the other, non-specific, playing a minor *rôle*.

In order to throw further light on the possible action of the serum in avian malaria the following experiments were carried out.

Experiment I.

" Immune " serum was obtained from canaries which, after recovery from an acute attack of malaria, had subsequently received a second injection of infected blood, from the effects of which they had recovered at least ten days previously. Blood from infected canaries, containing approximately 120 infected cells per 1000 red cells, was collected into hepatin, and dilutions of the blood in physiological saline, from 1 in 1 to 1 in 64, were made. Quantities of 0·15 c.c. of each dilution were mixed with 0·25 c.c. of " immune " serum and with 0·25 c.c. of normal canary serum. The mixtures were placed in the incubator at 37° C. for 1 hour and were then injected intraperitoneally into canaries. The birds were examined daily to determine the first appearance of parasites in the peripheral blood-stream. The results are shown in Table I. It will be seen that, though there may be considerable variation in the incubation period, there is, with the larger doses of infected red cells, no evidence of any sensitizing action on the part of the " immune " serum. With higher dilutions, however, there is a tendency for the incubation period to be lengthened in the birds inoculated with the " immune " serum as compared with those inoculated with normal serum. This experiment, therefore, suggests that contact with " immune " serum may render infected red cells more susceptible to phagocytosis.

TABLE I.—*Showing Lengthening of Incubation Period by " Immune " Serum.*

Dilutions of infected red cells.	Days from inoculation to appearance of parasites in the blood.	
	"Immune" serum.	Normal serum.
1 in 1	5–6	4–6
1 in 2	5–7	5–6
1 in 4	7–8	6–7
1 in 8	8 →	6–8
1 in 16	9–10	6–9
1 in 32	10 →	7 →
1 in 64	10 →	7–10

Three canaries were inoculated with each dilution.

Experiment II.

Blood was removed from four canaries on the first day of the appearance of parasites in the peripheral blood-stream. After determining the number of infected cells per 1000 red cells, each sample was used to inoculate a test

canary. Further blood samples, obtained after the crisis, when the infection rate of the cells had fallen to approximately the same level as before, were likewise inoculated into test birds. The incubation periods in all the test canaries were noted.

The results are recorded in Table II. It will be seen that, as in the experiments recorded by Boyd (1925), there is a slight prolongation of the incubation period in the birds inoculated with parasites taken at the end of an infection. It is possible that in canaries which have become tolerant to infection the red cells are acted upon by some serological mechanism which makes them more susceptible to phagocytosis by the cells of the reticulo-endothelial system.

TABLE II.—*Showing Incubation Periods in Canaries Inoculated with Blood taken at the Beginning and the End of an Infection.*

No. of original canary.	Incubation period in days of canaries inoculated with blood taken at beginning of infection.	Incubation period in days of canaries inoculated with blood taken at end of infection.
I	3	6
	4	4
	3	5
	5	8
II	6	7
	4	7
	7	8
	4	10
III	3	6
	3	4
	4	6
	6	11
IV	4	5
	6	8
	8	9
	5	6
Mean	4·7	6·9

PHAGOCYTOSIS AND ELECTRIC CHARGE.

Further evidence in support of this hypothesis is forthcoming from a consideration of the relation existing between the degree of phagocytosis and the electric charge of the ingested particles. Such a correlation has been shown in the case of bacteria by Falk and Matsuda (1926) and Broom and Brown (1930); and in the case of sensitized sheep's erythrocytes by Lucké, Strumia, Mudd, McCutcheon and Mudd (1933). Brown (1933, *a*, *b*), in studying the electric charge of the red cells in bird malaria, found that, although there is no

apparent difference between the electric charge of infected and uninfected red cells, profound alterations in their charge may occur, under certain conditions, during the course of an attack.

Experiment III.

Determinations of the cataphoretic velocity of the red cells were made in a series of 24 canaries during an acute primary attack of avian malaria. The

FIGURE I.

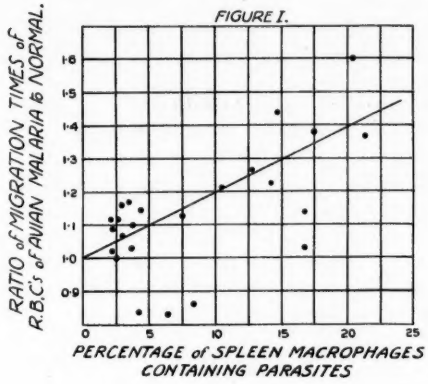
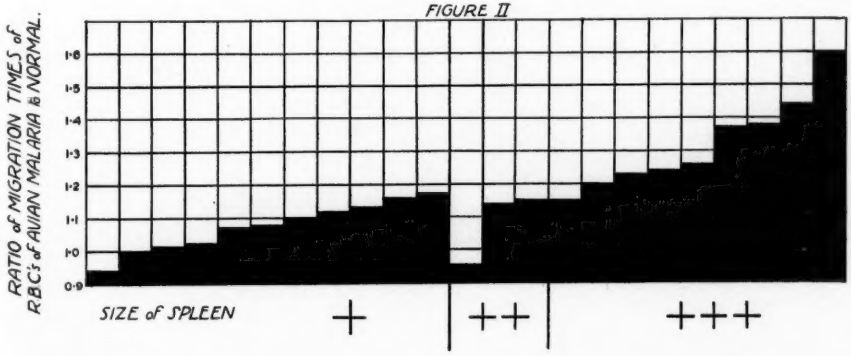


FIGURE II



birds, which had been kept under constant experimental conditions as regards food, etc., were killed immediately after making the determination. In each case the size of the spleen was noted, and smears from the spleen stained with Giemsa were examined microscopically. Five hundred macrophages were counted, and the percentage number noted that contained either infected red cells or what was obviously the debris of parasites; macrophages containing only malarial pigment were excluded. Fig. 1 shows the correlation between the degree of phagocytosis and the cataphoretic velocity of the red cells expressed as the ratio of the migration time to normal. Fig. 2 shows the

relation of the size of the spleen to the cataphoretic velocity of the red cells expressed as in Fig. 1.

From these results there is apparently a distinct correlation between the cataphoretic velocity of the red cells, the size of the spleen, and the degree of phagocytosis of infected red cells by the macrophages of the spleen.

DISCUSSION.

The experimental evidence here brought forward strongly suggests that in avian malaria the serum plays a very definite part in overcoming the infection. It is obvious, however, that the mechanism by which red cells infected with malaria parasites are removed from the peripheral circulation is by no means a simple one. It appears that at least three factors must be taken into account, one specific and two non-specific. Of the former little is known, but that it exists is shown by the experiments of Gingrich (1932), which demonstrated that little or no protection is afforded against one species of malarial parasite by tolerance acquired against another species. If the serum plays a part in this specific protective mechanism, it must obviously be a reaction between parasite and serum, and therefore, as a surface phenomenon, can probably only occur during the short extracellular stage of the parasite.

The two non-specific factors which play a part in the acquirement of immunity in avian malaria are (1) activation of the reticulo-endothelial system, (2) a serological change. The importance of the reticulo-endothelial system has been adequately emphasized by numerous observers. The importance of the serum is here stressed. The non-specific *rôle* of the serum has already been pointed out by Brown (1933, *b*), who showed that serum from a canary with a latent malarial infection is capable of reducing the electric charge not only of canary red cells, but of mouse red cells or even bacteria. He showed that this reduction in electrical charge is related to an increase in the euglobulin fraction of the serum, and is associated with the formation of a layer of euglobulin on the surface of the antigen, whether red cell or bacterium. In the case of bacteria, observations have also shown that changes in the amount of euglobulin present in the serum are related to alterations both in the electric charge of the bacteria and their phagocytosis rate.

The defence mechanism of the body is thus very similar in bacterial infections and in a protozoal disease such as avian malaria.

CONCLUSIONS.

(1) Serum from canaries with a latent malarial infection can be shown to have a sensitizing action on infected red cells, provided that the number of infected red cells is small in proportion to the amount of sensitizing serum.

(2) Blood from a canary which is recovering from an acute primary attack of malaria produces infection less readily than blood from the same canary at the beginning of the attack, the number of infected red cells being approximately the same at both times.

(3) The electric charge of the red cells is shown during an attack of avian malaria to be correlated with the size of the spleen, and with the phagocytosis rate of infected red cells by the macrophages of the spleen.

Our thanks are due to our colleague, Dr. J. C. Broom, for his kindness in calculating the regression coefficient.

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SOME FACTORS PREDISPOSING TO INFECTION BY *VIBRION SEPTIQUE* FROM THE ALIMENTARY TRACT: AN EXPERIMENTAL STUDY.

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Received for publication May 3rd, 1934.

IN a previous communication (Borthwick, 1933) on the experimental results in laboratory animals of enteral administration of staphylococcal exotoxin, it was shown that toxic effects were strictly determined by the hydrogen-ion concentration of the stomach and intestinal contents.

In recent years attention has been drawn to alimentary infections in sheep by various sporing anaërobes which are pathogenic by virtue of their exotoxins, and the question arises to what extent these infections and the resulting intoxications are dependent on the pH of the stomach and intestinal contents and, also, other local conditions. It has been shown by Bennetts (1932), in a study of infectious entero-toxæmia in sheep, that the natural disease could be reproduced by the administration of *B. ovitoxicus* culture, provided that secretion was inhibited and peristalsis reduced by the administration of opium and belladonna. This result, however, could be obtained either when the reaction of the gastric contents was left unaltered, or when a more alkaline reaction was produced in the stomach by the introduction of sodium bicarbonate.

It was therefore decided to carry out experiments in guinea-pigs on the enteral administration of certain organisms of the group of sporing anaërobes and their toxins, following the procedure of the author's previous work with staphylococcal toxin. In the first series of observations to be recorded in this paper *Vibrion septique* was used in view of its causal relationship to braxy or bradsot (Gaiger, 1922, *b*). It is generally agreed that in this infection the initial lesion is in the mucosa and submucosa of the abomasum, the fatal effect being essentially due to absorption of the exotoxin of the organism (Nielson, 1897; Gaiger, 1922, *a*). The factors determining the occurrence of the abomasal infection are still obscure, though external cold and the ingestion of frozen grass have been regarded as predisposing to the disease (Nielson, 1897; Hamilton, 1901; Gaiger, 1922, *a*).

METHODS.

Strains.

Two strains of *Vibrion septique* were used in the experimental work. One, "A7M", had been isolated from a case of braxy and was obtained from the Moredun Institute of the Animal Diseases Research Association. The second, "Bedford", was obtained from the National Collection of Type Cultures, and had originally been isolated from an amoebic abscess of the brain in the human subject.

Concentration of Cultures.

In order to reduce the volume introduced into the stomach, the fluid portion of an 18 to 24 hours' culture in cooked-meat medium was decanted and centrifuged, after which the supernatant fluid, except for 2 to 3 c.c., was discarded. The deposit of organisms, mixed with the remaining fluid, was employed in the feeding experiments.

Toxin.

Toxin was prepared according to Robertson's (1920) method, and its potency estimated by intracutaneous inoculation into the depilated skin of the abdomen of guinea-pigs, 0·3 c.c. of varying dilutions of the toxic filtrate being injected. The hydrogen-ion concentrations of the filtrate and the saline diluent were adjusted to pH 5 and the mixtures kept for 2 hours at room temperature before injection. This H-ion concentration is shown later to be favourable to the activity of the toxin. Control inoculations of saline at pH 5 and the filtrate, heated at 60° C. for half an hour, were also made. Necrosis at the site of inoculation, which was observed most readily after 48 hours, was the criterion of a positive result.

Administration of Toxin or Cultures to Guinea-Pigs.

Toxin preparations or concentrated cultures were introduced directly into the stomach by means of a syringe attached to a No. 3 rubber catheter. In experiments in which the hydrogen-ion concentration of the stomach contents was adjusted, a sample of the contents was aspirated, after introduction of the

organisms or toxin, and its reaction tested by means of a B.D.H. field capillator and a suitable indicator. The normal reaction varied from pH 3 to pH 5. Frequently, feeding caused a rapid secretion of acid, so that a 5 per cent. solution of sodium bicarbonate was introduced into the stomach in order to adjust the reaction to the range pH 5–6 or pH 7·6–7·8. After a short interval a further specimen was aspirated, and the hydrogen-ion concentration was again tested and adjusted if necessary. This procedure was repeated until samples gave the required reaction.

EXPERIMENTAL.

Vibrion septique cultures and toxin very rarely set up an infection or intoxication when fed to normal guinea-pigs. Observations were therefore carried out to ascertain the conditions which predispose to infection or toxic action.

The following conditions were investigated: (1) The optimal hydrogen-ion concentration for toxic activity. In culture medium pH 7·6 to pH 7·8 has been accepted as the optimal concentration for toxin production (Robertson, 1920; McEwan, 1926). (2) The inhibition of peristalsis. Since narcotine has been shown to exert a depressant action on the movements of the whole gastro-intestinal system in cats (Chopra, Mukhjee and Dikshit, 1930; Dikshit, 1932), it was thought of interest to determine whether this drug had a similar effect in guinea-pigs and, if so, to study the effect of administering *Vibrion septique* and its toxin when the alimentary tract was in a state of decreased activity. (3) The influence of cold, which has been acknowledged to predispose to braxy. The effect of cold on the movements of the alimentary canal was also investigated.

The Influence of pH on the Activity of the Toxin.

Intracutaneous inoculations, such as those described for determining the potency of the toxin, were employed to estimate the influence of pH on its

TABLE I.—*Results obtained on Intracutaneous Inoculation in Guinea-pigs of 0·3 c.c. Amounts of Dilutions of Toxin Adjusted to Varying H-ion Concentrations.*

pH.	Toxin dilutions. 1/2.	1 4.	1 8.	1/16.	1/32.	1/64.	Controls. Saline.	Toxin heated to 60° C. for ½ hour.
4	+	+	—	—	—	—	—	..
5	++	++	++	++	++	—	—	..
6	++	++	++	++	+	—	—	..
7	++	++	++	++	—	—	—	..
8	++	++	++	—	—	—	—	..
9	—	—	—	—	—	—	—	..
								—

— No reaction at site of injection. + A slight necrotic reaction at site of injection.
++ A considerable necrotic reaction at site of injection.

activity. Toxin and saline diluent were adjusted to various H-ion concentrations ranging from pH 4 to pH 9. Dilutions from 1/2 to 1/64 were made at each pH and kept at room temperature for 2 hours before being injected. Control inoculations of toxin heated to 60° C. for half an hour and of saline at each pH were made. These tests were repeated five times, and on each occasion controls failed to show any reaction. In all cases the most favourable H-ion concentration for the activity of the toxin was found to lie between pH 5 and pH 6. A typical example of the results obtained is given in Table I.

The Rate of Passage of Meals in the Alimentary Tract of the Guinea-pig.

In preliminary experiments methylene-blue thickened with gum acacia was fed to (*a*) normal guinea-pigs, (*b*) guinea-pigs which had received intramuscular injections of narcotine chloride 1 hour previously (3 mgm. of narcotine per kg. of body-weight). The animals were killed 15 minutes later, and it was observed that in normal guinea-pigs the dye had passed as far as the junction of the small and the large intestine, whereas in the treated animals it had not passed out of the stomach.

More detailed investigations were made by observing the rate of passage of barium meals by X-ray screening in (*a*) normal guinea-pigs, (*b*) guinea-pigs treated with narcotine, and (*c*) guinea-pigs chilled by exposure to a temperature of approximately 5° C. for about an hour in the open or, when the weather was too warm, by exposure to a temperature of 0° C. in the refrigerator for a short time.

Four normal guinea-pigs were given barium meals and examined by X-rays immediately after feeding, and at frequent intervals thereafter till all the meal had become diffused in the small intestine. The same animals were then treated with narcotine, fed with barium meals and examined by X-rays as before. The observations showed that narcotine in a dose of 3 mgm. per kg. of body-weight approximately doubled the time of passage.

Three guinea-pigs were chilled as described above. The normal rectal temperature varies from about 102° to 103° F. Chilling in the majority of animals produced only a slight fall in temperature (approximately 0·1° F.). A comparison of results obtained from normal and chilled animals showed no significant difference in the times required for a barium meal to pass through the stomach.

The results of these experiments suggest that narcotine is capable of arresting the movements of the gastro-intestinal system, and thus increasing the time of passage of meals, in guinea-pigs, whereas cold causes little difference in the rate of food passage.

Experimental Infection of Guinea-pigs.

The preparation and method of administration of *Vibrion septique* culture and toxin has been described. In view of the observations recorded it was decided to feed the following groups of guinea-pigs with concentrated culture:

Group 1.—Gastric contents adjusted to pH 7·6, this being the accepted H-ion concentration for the growth of *Vibrion septique* and the production of its toxin.

Group 2.—Gastric contents adjusted to pH 5, in view of the finding that this H-ion concentration is optimal for the maintenance of the activity of the toxin.

Group 3.—Treated with narcotine to decrease the movements of the alimentary canal. Two doses of narcotine were given ; 3 mgm. per kg. of body-weight followed by half this quantity 3–4 hours later.

Group 4.—Treated with narcotine, and gastric contents adjusted to pH 7·6.

Group 5.—Exposed to a low temperature.

Group 6.—Exposed to a low temperature, and the gastric contents adjusted to pH 7·6.

Group 7.—Concentrated cultures cooled to approximately 0° C. before administration. Concentrated culture was placed in a freezing mixture until it had just frozen solid. It was then removed, and as each 2 to 3 c.c. became sufficiently liquid it was administered to the guinea-pigs.

Group 8.—Controls.

Further control groups of guinea-pigs, in which the culture was not administered, were included : (1) Treated with narcotine, as in Groups 3 and 4 ; (2) exposed to cold, as in Groups 5 and 6.

TABLE II.—*Results Obtained from Experimental Feeding of Guinea-pigs with* Vibrion septique.

Group.	Number of animals.	Animals with definite evidence of infection and toxic effects.		Per cent. infected.	Survival times in days.
		Died.	Killed.		
1. Gastric contents adjusted to pH 7·6	21	1	1	9·5	11, 8
2. Gastric contents adjusted to pH 5	21	3	..	14	1, 3, 6
3. Treated with narcotine	21	15	2	81	2 to 19, except one which lived 42 days
4. Treated with narcotine and the gastric contents adjusted to pH 7·6	20	15	..	75	1 to 22
5. Exposed to a low temperature	20	9	1	50	3 to 19
6. Exposed to a low temperature and the gastric contents adjusted to pH 7·6	20	16	..	80	1 to 31
7. Culture administered at 0°C.	22	4	..	18	1 to 21
8. Controls	30	..	1	3·3	14

Additional controls :

6 guinea-pigs treated with narcotine remained in good health.

6 guinea-pigs exposed to a low temperature remained in good health.

The guinea-pigs which were killed had, apparently, begun to recover from infection.

Resistance to cold varied among the guinea-pigs ; some animals appeared chilled and sat huddled with raised hair, while others appeared to be unaffected. The experiments with Groups 5 and 6 were therefore repeated on the same animals on more than one occasion, so that the results might not be invalidated by feeding guinea-pigs in which cold had produced no effect.

One feature of interest observed was that neither the drug nor exposure to cold caused any noticeable alteration in the H-ion concentration of the stomach contents.

The summarized results of the experiments are given in Table II.

Post-mortem Findings.

These showed only slight variations. Care was taken to examine animals immediately after death, and when this was impracticable the possibility of post-mortem changes was taken into consideration in the histological examination.

Marked congestion and, usually, small hæmorrhages were present in the wall of the stomach ; the small intestine also showed acute congestion, which was occasionally associated with hæmorrhages. Histological examination showed that the hæmorrhages were caused mainly by rupture of capillaries in the submucosa. In addition, a considerable amount of atrophied epithelium was, generally, desquamated into the lumen.

The lungs were invariably intensely congested, and showed patchy or, occasionally, complete consolidation by infiltration with mononuclear cells. In some cases areas of both consolidation and emphysema were present.

The kidneys were intensely congested, and showed irregularly distributed areas in which the tubular cells had undergone degeneration. The stages of degeneration ranged from cloudy swelling to necrosis and, frequently, both the early and late stages were present in the same kidney. Infiltration with mononuclear cells and commencing hyalinization of the glomeruli were regularly found.

The suprarenals were usually enlarged and discoloured. Histological examination revealed a condition of acute congestion and the presence of diffuse degenerative changes in the cells of the medulla, some of which were necrotic.

Routine histological examination of the liver was not carried out. This organ was, however, occasionally investigated, when its mottled appearance and great friability indicated that it had undergone pathological alterations. The changes found in such cases were striking. Intense congestion and dilation of the veins were usual. In some instances lobulation had entirely disappeared, and the liver-cells showed fatty degeneration and, in some areas, necrosis. Where liver-cells could be distinguished the peripheral cells were found to be more affected than the central.

It is of particular significance that the histological findings indicated that all the pathological changes were of toxic origin.

Vibrion septique occurred constantly in the alimentary lesions and frequently in the lung lesions. On rare occasions it was isolated from the heart blood of animals which died within a few days of feeding.

The 5 guinea-pigs, recorded as killed in Table II, had been in poor condition

and losing weight; they then apparently began to recover and were killed and examined at this stage. In all five typical lesions were present.

The death of one guinea-pig from Group 5, not included in Table II, was attributed to a mediastinal abscess from which Gram-positive cocci were isolated. In addition to this infection, however, a lesion was present in the stomach mucosa from which *Vibrion septique* was isolated.

Administration of Toxin.

Toxin was administered in 8 to 10 c.c. amounts to guinea-pigs in the following groups, each of which contained 6 animals.

Group 1.—Gastric contents adjusted to pH 7·6.

Group 2.—Gastric contents adjusted to pH 5.

Group 3.—Treated with narcotine as described for the animals fed with culture, and gastric contents adjusted to pH 5.

Group 4.—Controls.

The animals in all these groups remained in good health and showed no signs of toxic effects.

DISCUSSION.

The preceding experimental data demonstrate certain conditions which predispose to *Vibrion septique* infection in guinea-pigs on enteral introduction of culture. The H-ion concentration of the gastric contents is not, either by itself or associated with other factors, of special importance. When, however, administration of drugs has caused diminished activity of the gastro-intestinal tract or when animals have been exposed to a low temperature, infection with *Vibrion septique* can be set up by intra-gastric administration of culture. The apparent factor influencing the onset of infection in the drug-treated animals is the opportunity to penetrate the mucosa of the stomach and small intestine afforded to the organisms by the increased time they are retained in this situation. These results agree with Bennetts's (1932) findings on the production of entero-toxæmia in sheep by administration of *B. ovotoxicus* cultures. Bennetts suggested that constipation is, therefore, an important factor in determining the onset of entero-toxæmia. If this is so, stasis would have a similar influence in the production of infection by *Vibrion septique* from the alimentary tract.

Cultures at room temperature, introduced into the stomach of guinea-pigs which have been exposed to a low temperature, set up an infection more readily than cultures at approximately 0° C. administered to normal animals. This suggests that the influence of external cold, rather than the ingestion of frozen food, is an important factor in *Vibrion septique* infection.

The findings reported here, therefore, suggest that two factors may be at work in producing alimentary infection in guinea-pigs: (1) Stasis of the stomach and intestine; (2) low temperature, which is more effective when applied to the animal as a whole than when applied locally to the mucous membrane of the alimentary tract.

Examination of guinea-pigs which died as a result of infection revealed the presence of extensive toxic changes in the internal organs. This finding

strongly indicates that death is primarily due to intoxication. Toxic filtrate, however, when introduced into the stomach of guinea-pigs, was unable to set up a pathological condition. This suggests that the toxin is not readily absorbed from the intact mucosa of the gastro-intestinal tract.

SUMMARY AND CONCLUSIONS.

(1) Hydrogen-ion concentrations within the range pH 5 to pH 6 are most favourable for the maintenance of activity of *Vibrion septique* toxin.

(2) Narcotine has a depressant influence on the movements of the gastro-intestinal system of guinea-pigs.

(3) Exposure of the guinea-pig to a low temperature (0° C.–5° C.) produces no alteration in the movements of the alimentary canal.

(4) The H-ion concentration of the gastric contents is of little importance in determining the production of a *Vibrion septique* infection from the alimentary canal.

(5) Animals in which alimentary activity has been reduced by the drug narcotine frequently show evidence of infection by *Vibrion septique* after intra-gastric administration of culture.

(6) Exposure of guinea-pigs to a low temperature, before administration of culture, predisposes to infection by *Vibrion septique*. Culture at approximately 0° C., introduced into the stomach of normal animals, does not readily cause infection.

(7) The general character of the changes found in the internal organs on post-mortem examination indicates the presence of a toxæmic condition in animals infected by *Vibrion septique*.

(8) Intra-gastric administration of toxin alone to guinea-pigs, whether normal, with the gastric contents adjusted to pH 5 or pH 7·6, or treated with narcotine, does not produce intoxication. The toxin is apparently not absorbed from the lumen of the stomach.

I wish to express my gratitude to Dr. C. M. Scott for his guidance in the pharmacological investigations. The work was carried out during the tenure of a Carnegie Scholarship, and I am indebted to the Moray Fund of Edinburgh University for a grant for experimental animals.

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THE IMMUNIZING ACTION OF EXTRACTS OF PNEUMOCOCCI (TYPES 1 AND 2) IN MICE AND RABBITS.

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Received for publication May 10th, 1934.

SOLUBLE specific substances were isolated from the various types of pneumococci by Heidelberger and Avery (1923, 1924), and from a number of other organisms by Zinsser and Parker (1923). These substances, shown to be complex carbohydrates, reacted in high dilution with their corresponding type antisera, but were found to be themselves incapable of exciting antibody formation on injection in animals. Later workers, in particular Perlzweig *et al.* (1923, 1925), Schiemann *et al.* (1927, 1931), Wadsworth and Brown (1931, 1933), and Felton (1932), obtained, from pneumococci, carbohydrate fractions which were capable of inducing active immunity in mice. Recently Avery and Goebel (1933) have isolated from Type 1 pneumococci, by acid extraction, an acetyl polysaccharide which differs chemically from the original soluble specific substance of Heidelberger and Avery by containing an acetyl grouping. This acetyl polysaccharide was found to produce an active type-specific immunity in mice. Its antigenic activity was dependent on the presence of the acetyl grouping, as the removal of this group by alkali resulted in loss of the power to immunize mice, and the resulting product was chemically and immunologically identical with the soluble specific substance as first isolated. The soluble specific substance is now designated deacetylated polysaccharide. They report, however, that they were unable to produce agglutinins, precipitins or protective antibodies by injection of the acetyl polysaccharide in rabbits, despite the use of a wide range of doses. This acetyl polysaccharide, therefore, differs in this respect from the active antigen of the heat killed intact pneumococcus. Day (1928, 1930, 1933), however, obtained extracts, containing substances which produced type-specific agglutinins in rabbits and active immunity in mice, from pneumococci, streptococci, staphylococci and organisms of the typhoid group. These substances, believed to be non-protein in nature, he designated type-specific antigens.

The present paper deals with the immunizing action of extracts of pneumococci (Types 1 and 2) containing these type-specific antigens of Day. It has been found that the extracts produce active antibacterial immunity in mice and agglutinins and protective antibodies in rabbits, equal in amount to those obtained by the use of similar doses of whole vaccine (heat-killed intact pneumococci).

EXPERIMENTAL METHODS.

Cultures.—One strain of type 1 pneumococcus and one of type 2 were used for the preparation of immunizing substances and for infection of mice.

These are the same strains that Day used, and their virulence is such that 2×10^{-8} c.c. of a 17-hour broth culture (containing approximately 10 cocci) regularly kills mice in less than 3 days. Frequent mouse passage was used to maintain virulence; stock cultures were made in cooked meat medium from the heart blood, and were stored at 5° C.

Preparation of vaccines.—Subcultures were made from the stock tubes into 2 per cent. rabbit serum broth. They were incubated at 37° C. for 17 hours, when they were enriched by the addition of half their volume of glucose broth and incubated for another 2 hours. The pneumococci were collected by centrifuging, resuspended in saline equal in volume to that of the original culture, and heated to 60° C. for 50 minutes.

Preparation of the type-specific antigen extracts.—Cultures were made as described above, the organisms were collected and resuspended in one-third the original volume of saline and an equal volume of N/10 HCl was added. The acid suspension was heated to 60° C. for 50 minutes and was then thoroughly centrifuged. The supernatant fluid was exactly neutralized with N/10 NaOH and the volume made up with saline to that of the original culture. This neutralized supernatant fluid contains the type-specific antigen (Day) and constitutes the type-specific antigen extract. Though it is not claimed that this method gives a supernatant completely free from bacterial debris, it was clearly demonstrated by Day (1928), when using inefficient extraction methods, that the fluids did not contain sufficient formed bacteria to produce any immunity in mice or rabbits. He found that the filtration of small quantities of fluid was impracticable, as the active antigen was adsorbed by the filter.

Immunization and testing of mice.—Mice were given one intraperitoneal injection of 0·3 c.c. of the type-specific antigen extract or of vaccine. Their immunity to the living organisms was tested 7 days later, using serial dilutions in broth of 17-hour serum broth cultures, injected intraperitoneally in 0·2 c.c. quantities.

Immunization of rabbits and determination of agglutinins and protective antibodies.—Rabbits were given a series of 5 intravenous injections of type-specific antigen extract or of vaccine, at weekly intervals. The doses were 2, 4, 6, 8, and 10 c.c. The animals were bled 7 days after the last injection; the blood was allowed to clot and placed in the ice-chest, the serum being collected the following morning.

Agglutination was performed with young broth cultures. The serum suspension mixtures were incubated at 50–55° C. for 2 hours and then placed in the ice-chest, readings being made the next morning. Protective antibodies were demonstrated by injecting groups of mice with 0·5 c.c. of serum intraperitoneally and testing them against serial dilutions of the living organisms the following day.

RESULTS OF THE IMMUNIZATION OF MICE.

It was found that the immunizing effect of one dose of 0·3 c.c. type-specific antigen extract was equal to that produced by the same quantity of whole vaccine. A very complete immunity resulted from one injection of type 1 preparations and less complete immunity from type 2 preparations. A

comparison of the activity of equivalent quantities of vaccine and of type-specific antigen extract of type 1 pneumococci is shown in Table I. The immunizing antigen of the vaccine, prepared by resuspending young, intact pneumococci in saline and killing by heat, is contained in the body of the organisms. The process of acid extraction results, therefore, in the liberation of active antigen into solution. Table II illustrates the type-specific nature of the immunity produced by the injection of a type 2 extract.

TABLE I.—*Comparison of the Immunity Produced in Mice by Pneumococcus (Type 1) Vaccine and by Type-specific Antigen Extract.*

Test dose of pneumococci (type 1).	Mice receiving one injection of 0·3 c.c. of—		Control mice (untreated).
	Vaccine.	Extract.	
10 organisms	S	S	D 2
10^2 ,,	S	S	D 2
10^3 ,,	S	S	D 2
10^4 ,,	S	S	..
10^5 ,,	S	S	..
10^6 ,,	S	S	..
10^7 ,,	S	S	..
10^8 ,,	D 1	D 1	..

S. = survival. D. = death. The number following indicates survival time in days after the injection of the test dose.

TABLE II.—*The Type-specific Nature of the Immunity in Mice following the Injection of One Dose of Type-specific Antigen Extract of Type 2 Pneumococci.*

Test dose of pneumococci.	Mice receiving one injection of 0·3 c.c. type 2 extract and tested with pneumococci.		Control mice (untreated) tested with pneumococci.	
	Type 2.	Type 1.	Type 1.	Type 2.
10 organisms	S	D 2	D 2	D 2
10^2 ,,	S	D 2	D 2	D 2
10^3 ,,	S	D 2	D 3	D 2
10^4 ,,	S	D 2	..	..
10^5 ,,	S	D 1	..	..
10^6 ,,	D 2	..	..	..
10^7 ,,	D 2	..	..	..

The Effect of Various Agents on the Immunizing Power of Type-Specific Antigen Extract in Mice.

Adsorption to globulin.—It was found that the whole of the immunizing substance could be removed from type-specific antigen extracts by adsorption. An acid extract of type 2 pneumococci was mixed with an equal volume of a saturated solution of ammonium sulphate; the mixture remained clear. A few drops of rabbit serum were added and the precipitate of globulin was collected by centrifuging and dissolved in saline. The solution was then neutralized with NaOH and the volume made up to that of the original culture with saline. The dissolved adsorbate and control untreated extract, in equal doses, produced the same degree of immunity in mice (Table III).

TABLE III.—*Removal of the Active Antigen from the Extract of Type 2 Pneumococci by Adsorption to Globulin.*

Test dose of pneumococci (type 2).	Mice receiving one injection of 0·3 c.c. of type 2 extract.		Control mice (untreated).
	Adsorbed to globulin.	Not adsorbed.	
10 organisms	S	S	D 2
10^2 ,,	S	S	D 1
10^3 ,,	S	S	D 2
10^4 ,,	S	S	..
10^5 ,,	S	S	..
10^6 ,,	D 2	D 2	..

Acid and alkali at 100° *C.*—Type-specific antigen extracts acidified to pH 4–5 and heated to 100° C. for 20 minutes retained their immunizing properties for mice, but were inactivated by alkali (pH 9–10) under these conditions.

RESULTS OF THE IMMUNIZATION OF RABBITS.

A course of five, weekly, injections of type-specific antigen extract, as described above, resulted in the production of type-specific agglutinins and protective antibodies (for mice) equal to those obtained by the injection of similar quantities of vaccine. Table IV details one experiment with Type 1 preparations.

TABLE IV.—*Comparison of the Type-specific Agglutinin and Protective Antibody Response in Two Rabbits Immunized* (A) *with Vaccine, and* (B) *with Extract of Type 1 Pneumococci.*

Agglutination Titres for Type 1 Pneumococci.

	Rabbit A serum.	Rabbit B serum.
Before immunization	0	0
After ,,	1/12·5	1/25

Protective Action of Sera for Mice. Animals Received 0·5 c.c. of Serum Intraperitoneally and were Tested against Living Organisms the Following Day.

Test dose of pneumococci (Type 1).	Serum A.	Serum B.	Control mice (untreated).
10 organisms	S	S	D 2
10^2 ,,	S	S	D 2
10^3 ,,	S	S	..
10^4 ,,	S	S	..
10^5 ,,	S	S	..
10^6 ,,	S	S	..

Survivors were not immune to type 2 pneumococci. The sera showed no agglutination with type 2 pneumococci.

DISCUSSION.

The foregoing experiments demonstrate that the type-specific antigen extracts of Types 1 and 2 pneumococci produce active type-specific immunity

in mice and type-specific agglutinins in rabbits, equal to those obtained by the use of similar doses of whole vaccine consisting of heat-killed young pneumococci in saline, in which the active antigen is present in the bodies of the organisms. These results are in agreement with the work of Day (1930, 1933). The active principles of these extracts, designated "type-specific antigens" (Day), appear, therefore, to be closely similar in antigenic properties to the immunizing antigen of the intact pneumococcus. Though these type-specific antigens have not yet been isolated and examined chemically, it seems probable that they are more complex in structure than the acetyl polysaccharide isolated from pneumococcus type 1 by Avery and Goebel (1933), since these workers failed to obtain any antibody response in rabbits with the polysaccharide. The action of alkali at 100° C. inactivates both the type-specific antigen and the acetyl polysaccharide. The exact relationship of the type-specific antigen to the acetyl polysaccharide remains to be determined, but it appears that the type-specific antigens are more closely allied to the immunizing antigens of the intact pneumococcus.

SUMMARY.

Extracts of pneumococci (Types 1 and 2), made by the action of N/20 HCl at 60° C. for 50 minutes, produce active type-specific immunity in mice and type-specific agglutinins and protective antibodies in rabbits.

Heating the extracts to 100° C. at pH 4–5 does not impair their immunizing action for mice, but similar treatment at pH 9–10 inactivates them.

The relationship of the active principles of these extracts to the immunizing antigen of the intact pneumococcus and to the acetyl polysaccharide of type 1 pneumococcus (Avery and Goebel) is discussed.

I wish to express my gratitude to Prof. H. B. Day, of the School of Medicine, University of Cairo, for his generosity in presenting me with his cultures, laboratory notes, etc., on his departure to Egypt. I wish, also, to thank Dr. John Freeman, Director of the Asthma Clinic, St. Mary's Hospital, for his advice and criticism, and the Asthma Research Council for a research grant.

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EXPERIMENTS WITH THE "O" ANTIGEN OF *CLOSTRIDIUM ŒDEMATIS MALIGNI* (*VIBRION SEPTIQUE*).

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Received for publication May 11th, 1934.

DESPITE the extended employment of formolized whole culture vaccines (anacultures) in the control of *C. œdematis maligni* infections in animals, it is remarkable that no detailed knowledge exists concerning the prophylactic value of the bacillary bodies contained in such anacultures. The researches of Leclainche and Vallée (1925), McEwen (1926) and others have indicated that the whole culture vaccine has a protective value greater than that afforded by the toxoid or toxin-antitoxin methods of immunization. Whether this enhanced value is due to the presence of bacterial cells, or to the use of a more potent toxoid, is not clearly determined.

In *C. chauvœi* prophylaxis it is established that the bacillary body ("O" antigen) is a powerful immunizing agent, and recent work (Henderson, 1932, *b* and *c*) has suggested that in this case, where true toxin production is still doubtful, the "O" antigen or autolysed products of the bacterial cell act as the principal antigenic elements of *C. chauvœi* vaccines. With *C. œdematis maligni*, however, where a potent toxin is produced and where an antitoxic immunity can be established against natural infection, the value of the "O" antigen in active immunization remains undetermined. Robertson and Felix (1930) have shown that a pure antibacterial mechanism can be established against *C. œdematis maligni* infection. Using a pure anti-"O" horse-serum, free from antitoxin, they were able passively to immunize mice against massive doses of an activated spore suspension of this organism.

If it were established that the bacillary antigen, freed from antitoxin, was a powerful agent in stimulating active immunity, then the enhanced value claimed for formolized whole culture vaccines might be ascribed to the "O" antigen present. Further, should the results be positive, it would be essential to determine the antigenic relationship existing among strains of *C. œdematis maligni* since, if variation occurred, it would indicate the use of polyvalent antigens for the preparation of anacultures.

The present investigation, therefore, is concerned with the antigenic value of the bacillary ("O") antigen of *C. œdematis maligni* in active immunization, and also with the antigenic relationship of various strains of this organism as estimated by analyses *in vitro*.

EXPERIMENTAL METHODS.

The technique adopted was essentially that developed for experiments with *C. chauvœi* (Henderson, 1932, *b*).

The "O" antigens, used for immunization, were prepared from 14–16-hour cultures in 1 per cent. glucose broth. The deposits, obtained by centrifuging, were twice washed in a large volume of distilled water. They were resuspended in distilled water to an opacity ten times greater than Burroughs Wellcome standard opacity tube No. 10, steamed for 2 hours at 100° C., and finally adjusted to a salt concentration of 0·85 per cent. with 10 per cent. NaCl solution.

Guinea-pigs were the experimental animals used. They received varying subcutaneous injections of the undiluted "O" antigen, and were tested, 14 days after the last inoculation of vaccine, with an activated spore suspension, given intramuscularly at a site opposite to that used for vaccination.

Suspensions used for infection experiments were prepared from 48-hour Dorset-egg growths and treated as previously described (Henderson, 1932, *b*), except that all washings were carried out in distilled water. Before use, the suspension was heated for one hour at 75° C., centrifuged and, finally, resuspended to the original opacity in saline. The test dose was given in a total volume of 0·6 c.c., and contained (1) 0·3 c.c. of the spore suspension in the required dilution, (2) 0·3 c.c. of a freshly prepared 5 per cent. solution of $CaCl_2$ in distilled water. In each experiment an indication of the relative infecting power of the spore suspension used was obtained by titrating the test dose, and higher dilutions of it, in normal guinea-pigs.

PRELIMINARY ANIMAL EXPERIMENTS.

In the early experiments a method was adopted identical with that used for the successful immunization against *C. chauvœi* infection. Guinea-pigs, of approximately 250–300 gm. weight, received 5–6 c.c. of the undiluted "O" antigen, given subcutaneously in 3 doses at 4–5-day intervals. They were tested 14 days after the last inoculation with an activated spore suspension prepared from the homologous strain.

The results obtained by this method were disappointing, as only traces of acquired immunity could be demonstrated. For example, in one experiment, of five vaccinated animals which received a test dose ten times larger than the smallest certain infecting dose for normal guinea-pigs, only one animal survived after exhibiting signs of severe illness and developing a severe local gangrene, from which it completely recovered. Three other animals received the smallest certain infecting dose of spores and two survived without symptoms. Death, in all cases, was confirmed as due to *C. œdematis maligni* infection.

For later experiments, therefore, the method of immunization was modified by substituting for one series of 3 inoculations of vaccine two series of 3 inoculations each, the series being separated by an interval of about 14 days. Each animal received by this method 12 c.c. of "O" antigen, given in 6 doses. Guinea-pigs of approximately 250–300 gm. weight were selected, and no

ill-effects on their general health were observed as a result of the massive dose of antigen given. Occasional swellings were noted over the site of inoculation, and in some instances the superficial inguinal glands became very enlarged when the animals received the test dose.

RESULTS OF EXPERIMENTS WITH GUINEA-PIGS.

Table I illustrates a typical result obtained by this latter method of immunization. The table also includes, for comparative purposes, the results obtained in a parallel experiment using the earlier method. The antigen used was prepared from the strain " Pasteur III ", and the test spore suspension was prepared from the same strain.

TABLE I.—*Active Immunization of Guinea-pigs with " O " Antigen.*

Dilution of infecting suspension.	Vaccinated guinea-pigs.				Controls.	
	Double series inoculations. (12 c.c. vaccine.)		Single series inoculations. (6 c.c. vaccine.)			
	Tested.	Survived.	Tested.	Survived.	Tested.	Survived.
1 : 20	5	5	..	..	1	0
1 : 200	6	5	5	1	2	0
1 : 2,000	6	5	5	2	4	0
1 : 10,000	..	..	..	..	4	1

Both the vaccine and the infecting suspension were prepared from the strain " Pasteur ".

The vaccinated guinea-pigs were placed in 3 groups for testing, each group receiving a different dilution of the infecting suspension. The titration of this suspension in normal guinea-pigs showed that a spore dilution of 1 in 2000 gave the smallest certain infecting dose ; regular deaths occurred in from 17–24 hours with dilutions up to and including 1 in 2000. It is seen, therefore, that vaccinated animals received respectively 1, 10 and 100 certain infecting doses of spores.

The course of the infection in the vaccinated animals was, in many respects, similar to that obtaining in experiments with *C. chauvœi*. Of 17 guinea-pigs tested, 2 died of a typical *C. œdematis maligni* infection. Among the survivors 4 developed only small local œdematous swellings at the site of inoculation, which had subsided by the fourth or fifth day after inoculation. Eleven animals, however, showed definite signs of illness, commencing about 18 hours after infection and prolonged for a period of at least 48 hours. Severe perforating gangrene also developed (extending in some cases over the whole surface of the abdomen) in from 24–60 hours after infection. These gangrenes rapidly contracted, and in all cases they had healed completely within four weeks, but it was evident in most instances that there had been a complete necrosis of the local muscle tissue.

EXPERIMENTS ON CROSS-IMMUNIZATION.

Using the method of agglutination, Robertson (1920) has divided the various strains of *C. œdematis maligni* into at least four groups and, by an examination of further strains, Davesne (1928) and Weinberg and Mihailesco (1929) claim to have found at least six serological groups within the species. The technique used by these workers makes it probable that their grouping of the strains is based on the "H" or flagellar antigen and is not concerned with the "O" antigen relationship.

Felix and Robertson (1928), in their examination of the "H" and "O" antigens of the spore-bearing anaërobes, found that the "O" antigen of *C. œdematis maligni* exhibited a very considerable overlapping among the various strains examined, including representatives from the four "H" groups of Robertson. In later experiments (1930), however, the results of animal protection tests were not in consonance with such postulated overlapping. They showed that a monovalent "O" horse-serum, prepared against one strain of *C. œdematis maligni*, could not protect against infection with heterologous strains. In view of these findings it was decided to establish the relationship of the various strains by active immunization experiments.

Strains representative of the three principal groups of Robertson (1920) were used for the immunization of guinea-pigs by the method described above.

Table II records the results of one experiment, including three groups of guinea-pigs immunized respectively with "O" antigen prepared from strains (*a*) "Pasteur" (of human origin) representative of Group I, (*b*) "Milk" (of animal origin—cow's milk) representative of Group II, and (*c*) "Stokes" (of human origin) representative of Group III.

The infecting suspension used for testing all vaccinated animals was prepared from the strain "Pasteur", and the dilution giving the smallest certain infecting dose was 1 in 10^6. Guinea-pigs from each group were placed in three lots; those vaccinated with the homologous strain (Group I) antigen received respectively 50, 500 and 5000 certain infecting doses, while those in the groups vaccinated with heterologous antigens received respectively 10, 50 and 500 certain infecting doses.

TABLE II.—*Cross-Immunization Experiment.*

Dilution of infecting suspension "Pasteur" (Group I).	Guinea-pigs vaccinated with "O" antigen prepared from strains: "Pasteur" (Group I).		"Milk" (Group II).		"Stokes" (Group III).		Controls.	
	Tested.	Survived.	Tested.	Survived.	Tested.	Survived.	Tested.	Survived.
1 : 200	4	3	..	..	..	..	..	..
1 : 2,000	4	4	3	0	3	0	2	0
1 : 20,000	4	3	4	0	4	3	3	0
1 : 100,000	..	..	4	2	4	2	3	0
1 : 1,000,000	..	..	..	..	..	..	4	0
1 : 10,000,000	..	..	..	..	..	..	2	1
1 : 100,000,000	..	..	..	..	..	..	2	2

It is seen that of 12 animals receiving homologous strain antigen only 2 succumbed to *C. œdematis maligni* infection, 7 developed more or less severe local gangrenes, from which they recovered completely, and 3 showed only small local disturbance. Guinea-pigs vaccinated with strain "Milk" (Group II) antigen, however, exhibited only traces of an active immunity against this infection with a Group I strain. Nine of the 11 animals died from a typical infection, and 2 recovered after developing extensive local gangrene. On the other hand, the animals vaccinated with strain "Stokes" antigen showed a definite though incomplete immunity. Six died, while 5 recovered after exhibiting varying degrees of local gangrene.

Similar cross-immunization experiments, using strains from Groups II and III for infection, have given comparable results, and clearly establish the presence of specific "O" antigens among strains of *C. œdematis maligni*. They show, further, the existence of a common "O" antigen shared, in varying degree, by the different strains.

It appeared from this work that the specificity of "O" antigen ran parallel with the specificity of "H" antigen previously recorded by *in vitro* analyses, but later experiments have shown that such parallelism does not necessarily exist. For example, Table III records the results of one experiment including two batches of guinea-pigs immunized with strains which exhibit a high degree of specificity in "H" antigen. One batch was vaccinated with "O" antigen prepared from the strain "Jerral", representative of Robertson's Group IV, and the other received "Milk" (Group II) antigen. Half of the animals from each batch received an infecting suspension prepared from the strain "Jerral", and the other half were tested with a suspension prepared from the "Milk" strain. It is clear that complete cross-immunity was induced against infection with the heterologous strains, and the fact is thus established that the specificities of the "H" and "O" antigens do not necessarily run in parallel.

TABLE III.—*Vaccination with Heterologous Strains Identical in "O" Antigen.*

Strain used for infection.	Dilution of infecting suspension.	Guinea-pigs vaccinated with "O" antigen prepared from strains: "Jerral" (Group IV). Tested.	"Jerral" (Group IV). Survived.	"Milk" (Group II). Tested.	"Milk" (Group II). Survived.	Controls. Tested.	Controls. Survived.
"Jerral"	1 : 1,000	3	2	3	2	2	0
	1 : 10,000	3	3	3	3	4	0
	1 : 100,000	..	..	..	..	4	0
	1 : 1,000,000	..	..	..	..	2	1
"Milk"	1 : 200	3	3	3	3	2	0
	1 : 2,000	3	2	3	3	4	0
	1 : 20,000	..	..	..	..	4	0
	1 : 100,000	..	..	..	..	2	2

IN VITRO ANALYSES OF THE "H" AND "O" ANTIGENS.

The strains employed in the animal experiments have been used for the preparation of rabbit immune sera, and have been examined, together with other strains, by agglutination and complement-fixation methods.

Analyses by Agglutination Tests.

The methods adopted for the preparation of rabbit immune sera and the technique of the agglutination tests were essentially those used in the *in vitro* experiments with *C. chauvœi* (Henderson, 1932, *a*). As previously noted, it has been found essential to use separate suspensions for the ready differentiation of " O " and " H " agglutinins. Further, to detect low titres of " H " agglutinins it is necessary to use " H " sera from which the " O " antibody has been completely absorbed.

In the examination of the "H" antigen the production of agglutinins and direct and cross-agglutination were considered. Table IV shows the results of a cross " H " agglutination test, using 4 sera and 4 strains representative of the original groupings of Robertson. While it is shown that a specific "H" antigen is possessed by each group, there appears to be a common "H" antigen shared in low titre by at least three of the groups.

TABLE IV.—*Cross " H " Agglutination Test.*

Pure " H " sera (absorbed in dilution 1 : 100 with homologous suspensions heated for 2 hours at 100° C.).	Titre of " H " agglutination with " flagellar " suspensions of strains :			
	" Pasteur " (Group I).	" Milk " (Group II).	" Stokes " (Group III).	" Jerral " (Group IV).
" Pasteur " . . .	20,000	500	500	100
" Milk " . . .	200	2000	100	0
" Stokes " . . .	500	100	10,000	100
" Jerral " . . .	0	0	0	1000
Control :				
Normal rabbit serum .	0	0	0	0

Titre 0 = negative result in dilution 1 : 100. Controls with steamed " O " antigen showed that " O " agglutinins had been completely removed. Reading taken after 3½ hours (2 hours 37° C. and then at room temperature).

The " O " antigenic relationship among strains was investigated by cross-agglutination and agglutinin absorption tests. Table V shows the results of a cross " O " agglutination test with 4 sera and 4 strains. The specificity noted among certain strains (" Pasteur "—Group I—and " Milk "—Group II) in animal experiment is corroborated by these *in vitro* results, while the presence of a common " O " antigen is clearly shown by the overlapping of the strains " Pasteur " and " Stokes ". The complete identity of the " O " antigens of the strains " Milk " (Group II) and " Jerral " (Group IV), indicated by *in vivo* experiment, is again demonstrated. Table VI illustrates a typical result obtained in a cross-absorption test with 2 sera and 5 strains. The agglutinins are completely absorbed from the Pasteur serum by strains belonging to this group,* while the strain " Stokes " only gives partial absorption. On the other hand, the strains " Milk " and " Jerral " fail to effect any absorption. With the serum prepared from the strain " Milk " a reciprocal result is obtained.

* Strain KF was kindly supplied by Mr. Dalling, of the Wellcome Physiological Research Laboratories, and has been shown in these experiments to be identical with the strain "Pasteur". The "H" antigen composition of the strain is not yet known.

TABLE V.—*Cross "O" Agglutination Test.*

Rabbit "O" immune sera prepared from strains:	Titre of "O" agglutination with strains: "Pasteur" (Group I).	"Milk" (Group II).	"Stokes" (Group III).	"Jerral" (Group IV).
"Pasteur"	2000	10	50	10
"Milk"	10	1000	20	2000
"Stokes"	50	10	500	10
"Jerral"	10	2000	50	5000
Control: Normal rabbit serum	0	0	0	0

Titre 0 = a negative result in a dilution of 1 : 10. Suspensions used for agglutination steamed 2 hours at 100° C. in distilled water. Reading taken after 24 hours (4 hours at 45° C., then at room temperature).

TABLE VI.—*Cross Absorption Test.*

Agglutination with strains.	Serum dilutions.	"O" serum (strain "Pasteur") absorbed in dilution 1 : 100 with strains: "Pasteur." Opacities of absorbing suspensions: 10×5.	"Pasteur" 10×10.	"Milk." 10×10.	"Stokes." 10×10.	"Jerral." 10×10.	"K.F." 10×10.	"O" serum unabsorbed.
"Pasteur"	1/100	±	−	+++	+++	+++	−	+++
	1/200	−	−	+++	+++	+++	−	+++
	1/500	−	−	++±	++	++	−	++±
	1/1000	−	−	+±	+	+±	−	+±
	1/2000	−	−	±	−	−	−	±

Agglutination with strains.	Serum dilutions.	"O" serum (strain "Milk") absorbed in dilution 1 : 100 with strains: "Milk." Opacities of absorbing suspensions. 10×5.	"Milk." 10×10.	"Pasteur." 10×10.	"Stokes." 10×10.	"Jerral." 10×10.	"K.F." 10×10.	"O" serum unabsorbed.
"Milk"	1/100	−	−	+++	+++	−	+++	+++
	1/200	−	−	+++	+++	−	+++	+++
	1/500	−	−	++±	+++	−	++±	+++
	1/1000	−	−	+±	+±	−	±	+±
	1/2000	−	−	−	−	−	−	−

+++ to ± = varying degrees of agglutination observed by the naked eye. Suspensions for agglutination and absorption heated for 2 hours at 100° C. in distilled water at an opacity 10×10 (B.W. standard opacity scale). Sera absorbed 2 hours at 37° C. Readings taken after 24 hours (4 hours at 45° C., and then at room temperature).

Analyses by Complement-fixation Tests.

This method has been found to be readily applicable to the study of the spore-bearing anaërobes. The results obtained, not only in this series of experiments, but in work now in progress with *C. tetani*, indicate that it is worthy of extended use in this field.

For the test, a standard dose of amboceptor (5 M.H.D.) was used with varying M.H.D. of guinea-pig complement. The "O" antigen, which was prepared as for "O" agglutination tests, was tested, immediately before use, against 2 M.H.D. of complement; the dose used was one-fourth of the smallest amount which inhibited lysis. The test was conducted in a total volume of 1·5 c.c. The serum, complement and antigen mixture was incubated for 1 hour at 37° C., and 0·5 c.c. of a 3 per cent. suspension of sheep red cells was then added. Readings were taken after incubation for 1 hour at 37° C. and again after standing overnight at ± 1° C. In all cases non-specific controls were introduced to exclude the interference of non-specific factors.

Direct and cross complement-fixation tests were conducted with each "O" rabbit serum. Table VII, illustrating a typical experiment, shows that the "O" agglutinin titres recorded in Tables V and VI were confirmed by the complement-fixation method.

TABLE VII.—*Cross Complement-fixation Test with "O" Immune Sera.*

"O" immune sera prepared from strains.	Titre of complement-fixation with strains:			
	"Pasteur" (Group I).	"Milk" (Group II).	"Stokes" (Group III).	"Jerral" (Group IV).
"Pasteur"	1000	0	0	0
"Milk"	0	500	0	1000
"Stokes"	0	0	500	0
"Jerral"	0	1000	0	2000
Control:				
Normal rabbit serum	0	0	0	0

Titre 0 = a negative result in a dilution of 1 : 20. Test conducted in the presence of 2·5 M.H.D. guinea-pig complement and 5 M.H.D. of amboceptor. Controls: Antigens in 4 × concentration and sera in twice the amount used in the test gave complete hæmolysis.

DISCUSSION.

In any experiments designed to demonstrate the value of an antibacterial mechanism against a bacterial infection where potent exotoxins are produced, a particularly solid type of immunity must be developed. In *C. œdematis maligni* infection the cause of death is an acute toxæmia, following the multiplication of the organisms and the associated toxin production. Protection due to an antitoxic immunity depends solely on the neutralization of this accumulated toxin, the factor responsible for the continued vegetation of the organism being thus removed. In experiments on antibacterial immunity, therefore, where no antitoxic immunity is developed, it is essential that the primary infection should be strictly localized, as any extensive multiplication of the organisms with the associated toxin production will lead rapidly to a fatal issue. For this purpose massive doses of the immunizing agent had to be used, although such spartan methods of immunization are not likely to enter into practical prophylaxis. The absence of free toxin from the "O" antigens was controlled by mouse neutralization tests, using sera from rabbits hyperimmunized with the vaccines.

In a recent paper Roberts (1933) criticizes the method of testing antibacterial immunity by the use of a calcium-activated, toxin-free spore suspension, claiming that it is theoretically unsound (at least in *C. chauvœi* experiments), and that infection with " whole-culture " gives as regular and reliable results. If it is admitted that in natural infections the vegetation of spores is primarily independent of the presence of toxin, then an experimental infection should, if possible, simulate the natural infection in that respect. Moreover, in experiments on antibacterial immunity, the direct introduction of toxin would frustrate the primary function of the mechanism, which is intended to control vegetation and multiplication, and thereby to limit toxin production.

From the results obtained by animal experiment there is evidence of the development of a powerful antibacterial mechanism; this establishes the importance of the bacillary body in the formolized vaccines of veterinary practice. The experiments on cross-immunization indicate the use of a polyvalent antigen for the preparation of such vaccines, but the number of animal strains examined makes this inference tentative only, and it is considered that a further examination of strains is desirable.

A point of interest in the experiments with vaccinated animals submitted to a spore-calcium test was the appearance, in most cases, of a severe perforating gangrene followed by complete recovery. In non-vaccinated controls, however, the appearance of a perforating gangrene was invariably the precursor of a fatal issue. This suggests that local vegetation of spores takes place in vaccinated animals and, as a result of toxin accumulation, multiplication proceeds beyond the calcium chloride barrier. Only then is the antibacterial mechanism brought into action to produce the strict localization of the lesion. The nature of this mechanism is now being investigated.

The results of the *in vitro* experiments indicate that the " O " antigen functioning in agglutination and complement-fixation is directly responsible for the production of the protecting immune body, as the sharp differentiation of " O " antigens exhibited in the *in vivo* tests is corroborated by the *in vitro* findings.

It is believed that the methods used in these experiments and in those with *C. chauvœi* have introduced a useful technique for the classification of the spore-bearing anaërobes. As with the aërobes, these organisms may readily be classified on the basis of their " O " and " H " antigenic composition. With *C. œdematis maligni* it is suggested that the original grouping based on " H " antigenic composition should be replaced by a primary classification on the basis of the " O " antigen relationship, and the " H " antigen should form the basis of a secondary grouping.

SUMMARY.

(1) An active antibacterial immunity can be established against *C. œdematis maligni* infection by the use of pure " O " antigen.

(2) The immunity developed is type-specific. A common " O " antigen, however, is shared by strains in varying degree, and is effective in producing minimal degrees of cross-protection.

(3) Reaction to infection in immunized animals is generally associated with a severe local perforating gangrene.

(4) The "O" antigen functioning in agglutination and complement-fixation is directly responsible for the production of the protecting immune body.

(5) A classification of *C. œdematis maligni* strains is suggested. The basis of primary differentiation should be the "O" antigen relationship, and the "H" antigen content should form the basis of a secondary grouping.

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THE TREATMENT OF SEPTICÆMIA IN RABBITS WITH LYMPH-GLAND FIXATION ABSCESSES.

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Received for publication May 11th, 1934.

THE formation of sterile abscesses in the subcutaneous tissues as a therapeutic anti-infective measure has received considerable attention in France and Germany in recent years. Savy (1922) considered that these abscesses would operate by concentrating at a given point the microbes and toxins in the circulation, or by stimulating the leucocytes, or as an antiseptic by reason of the oxygenating properties of the substance injected. The substance used for injection by all investigators was essence of turpentine. Delore (1930) caused fixation abscesses in this manner in patients suffering from tuberculosis. In cases of acute and subacute tuberculosis he obtained no appreciable result; in chronic cases he observed a diminution in the production of sputum.

Le Guyon (1931) carried out a number of experiments to ascertain the value of sterile abscesses in infections. He inoculated guinea-pigs and mice with anthrax bacilli, virulent streptococci, pneumococci and tubercle bacilli, but found that in these animals turpentine fixation abscesses failed to prevent fatal results.

Recently I carried out a series of experiments on similar lines, but the animals used were rabbits, and the substance for producing the abscesses was lymph-gland instead of turpentine.

The objects of the experiments were—

1. To increase the leucocyte count.
2. To increase the bactericidal power of the blood.
3. To test the value of sterile abscesses in preventing or curing septicæmia, caused by injecting virulent streptococci intravenously into rabbits.

Strain of Organism.

The strain of streptococcus used was the Aronson-Wamoscher—the most virulent we know. The dose of Aronson-Wamoscher streptococci, given intravenously, was about 4,000,000 in 1 c.c. of normal saline. Dr. Leonard Colebrook found that a subcutaneous injection, into the ear, of about 2200 of the organisms killed a rabbit in 110 hours.

Method of Production of Sterile Abscesses.

I experimented with various dried extracts, including spleen, liver and lymph-gland, and found that the latter, when injected under the skin of the back of a rabbit, produces a fixation abscess, without any marked local inflammatory reaction such as occurs when splenic extract is used and without any apparent pain. The dried lymph-gland, being nearly insoluble, is suspended in distilled water; the dose is 0·3 gm. in 3 c.c. of water, and it is necessary to use a large-bore needle for the injection.

Nature of Abscess Produced by Injection of Lymph-Gland.

The abscess manifests itself as a soft swelling on the day after injection, and becomes fully developed in 7 to 10 days. Unless the pus is evacuated, it remains for months as a large hard nodule and then gradually disappears. When opened, the abscess is found to consist of thick pus, which is darkly stained, due to the residual pigment from the lymph-gland preparation. It has a yellow capsule about 1/10 in. thick and is sometimes multiple. The capsule is very friable and tears easily, but with careful dissection it can be removed intact from its bed in the subcutaneous tissue.

An abscess was examined microscopically by Dr. J. W. Orr, who reported as follows:

"The central part of the lesion consists of granular debris, evidently derived from the injected material, infiltrated with eosinophile and a few neutrophile polymorphonuclear leucocytes. Around this is an œdematous area thinly infiltrated with eosinophile leucocytes and histiocytes with long processes. Further out there is a zone consisting of newly-formed capillaries and young fibrous tissue, with numerous large irregular histiocytes and eosinophile leucocytes, and a few lymphocytes and neutrophile polymorphonuclears."

Other Effects Produced by Injection of Lymph-Gland.

Associated with the abscess there is, as a rule, an increase in the number of leucocytes in the blood, preceded however, during the first 7 to 10 days after injection, by a leucopenia. The increase in white cells is possibly due to stimulation of the bone-marrow itself.

Experiment.—An injection of 0·3 gm. of desiccated lymph-gland in 2 c.c. of sterile saline was given beneath the skin of a rabbit, and a second injection 2 days later. The leucocyte count rose from 3400 per c.mm. before the injection to 19,900 per c.mm. on the 7th day, the differential percentage count on the latter date being: polymorphonuclear leucocytes 49, lymphocytes 48, transitional cells 2, and eosinophile cells 1. Two fixation abscesses were present, the first large, the second rather smaller. On the 26th day of the experiment a third injection was given, and the leucocyte count fluctuated between 8500 and 13,200 per c.mm. during the next 2 months. The bactericidal power of the blood was tested by Dr. I. H. Maclean a month after the first injection; the serum killed 82 organisms out of 200, while a normal control killed 78, showing that there was very little increase in the bactericidal power of the blood by *in vitro* tests. The bactericidal power of pus from one of the abscesses was later tested by Prof. A. Fleming; he found that the pus did not inhibit the growth of organisms. The leucocyte count in other rabbits injected with lymph-gland did not show as marked an increase as in the above case, but it has been found that, following an initial leucopenia, there is usually a leucocytosis of from 25 to 100 per cent. or more, lasting over a prolonged period. It is claimed that nuclein, yeast, novarsenobillon, mercurial preparations, etc., are capable of stimulating the formation of leucocytes, but experiments with these substances showed that the leucocytosis produced by them was only a temporary phenomenon, as the leucocyte count fell to the normal level at the end of about 24 hours, and did not rise again afterwards.

I have no means of judging whether the results obtained from the subcutaneous injection of dried lymph-gland are due to leucocytic action, or if they are due to some substance in the lymph-gland itself. It is possible to obtain a substance from desiccated lymph-gland by extraction with alcohol. This substance appears in the form of a grey powder, and I was able to obtain 0·1 gm. of the powder from 42 gm. of the dried gland, which is equivalent to 283 gm. of the fresh gland. Injection of 0·01 and 0·02 gm. of the extract into a rabbit caused a slight increase in the leucocytes, but no abscess.

ANIMAL EXPERIMENTS WITH INJECTIONS OF LYMPH-GLAND AND STREPTOCOCCI.

Nine normal rabbits were used; each received, intravenously, doses consisting of approximately four million virulent Aronson-Wamoscher streptococci. Four of them served as controls; they received no treatment of any kind, and they all died. These controls were, as far as possible, rabbits of the same litter and of approximately the same weight as the animals of the experiment proper. Three of the latter were given subcutaneous injections of desiccated

lymph-gland, 0·4 gm. in 2·5 c.c. of sterile distilled water, from 5 to 10 days before the streptococcal injections. The other 2 received streptococcal injections first, followed 5 hours later by the gland injections. All 5 animals recovered completely. Thus, of 9 rabbits suffering from septicæmia 4 untreated controls died within a few days, while 5 treated with lymph-gland fixation abscesses recovered.

Protocols of the Experiments.

Rabbit No. 1.—Weight 1950 gm. White blood-cells 8400 per c.mm. Subcutaneous injections of 0·4 gm. of lymph-gland extract were given into each side. On the 5th day the rabbit received an intravenous injection of 200,000 streptococci, and on the 8th day a larger dose of 4,000,000 organisms. The white blood-count fluctuated between 8400 and 5200 per c.mm. until the latter date, but on the 10th day rose to 12,900 per c.mm., and the counts from the 10th to the 15th day ranged between 12,800 and 10,000 per c.mm. By the 15th day the rabbit had developed marked stiffness and swelling of one of the joints of the right fore leg, also some induration and thickening of the left ear, which was the one used for the streptococcal injections; its weight had dropped from 1950 to 1850 gm. It was, however, taking food well, and otherwise appeared quite fit. On the 17th day the leucocyte count was 10,000 per c.mm., and 10 days later 8000 per c.mm. The joint swelling gradually subsided, and 3 months later the animal had completely recovered, its weight having increased to 2400 gm. Six months after the injections all that was left of the sterile abscess was a nodule the size of a small marble, the white cell-count was 10,000 per c.mm., and there was a faint cloud of albumin in the urine.

The untreated control, which received the same doses of streptococci on the same days, died from septicæmia 4½ days after the second injection.

Rabbit No. 2.—Weight 1930 gm. White blood-cells 9600 per c.mm. Two injections of 0·4 gm. of lymph-gland were given, one into each side. White blood-count on 10th day 17,000 per c.mm. On the 11th day 4,000,000 streptococci were injected. Abscesses on back were very large. On the 20th day white blood-count was 10,500. Weight 1520 gm. Joints stiff, and trace of albumin in urine. The animal recovered completely in 3 months.

Control (same litter), weight 2270 gm.; received 4,000,000 streptococci and died in 4 days.

Rabbit No. 3.—Weight 2520 gm. White blood-cells 6700 per c.mm. Lymph-gland injections into each side. White blood-count rose to 17,200 per c.mm. 9 days later. Intravenous injection of 4,000,000 streptococci on 11th day. On the 16th day joints were swollen and stiff. White blood-count 16,800 per c.mm. Loss in weight of 170 gm. Blood-culture was sterile, while culture from control rabbit showed a number of colonies of streptococci. Leucocytosis continued for a month, and the rabbit recovered completely in 33 days.

Control, weight 2750 gm.; received 4,000,000 streptococci; lost 370 gm. in weight and died in 11 days.

Rabbit No. 4.—Weight 2420 gm. White blood-cells 4600 per c.mm. Streptococcal injection, followed 5 hours later by lymph-gland injection into each

side. On the 12th day the joints were stiff. White blood-count 17,900 per c.mm. Some albuminuria. The animal lost 900 gm. in 3 weeks, but put on weight again and had completely recovered in 3 months.

Control (from the same litter) died in 7 days.

Rabbit No. 5.—Weight 2700 gm. White blood-count 4600 per c.mm. The usual streptococcal injection was given, followed 5 hours later by one lymph-gland injection. White blood-count 14,809 per c.mm. on 3rd day. A second lymph-gland injection was given on the 6th day. Dr. I. H. Maclean at this stage found streptococci in the animal's blood, but none in that of the control, which died a few hours later—the death being probably due to intense toxæmia rather than to septicæmia. Rabbit No. 5 at the end of 3 weeks was running about with an enormous fixation abscess on its back, which on culture proved quite sterile. It lost only 360 gm. in weight, and completely recovered in less than a month. Three months later a test of its blood showed marked increase in the bactericidal power, as compared with the blood of a normal rabbit.

Control (from a different litter) was also the control for Rabbit No. 4. It died in 7 days.

SUMMARY.

Fixation abscesses, obtained by the subcutaneous injection of desiccated lymph-gland, were used for the treatment of septicæmia in rabbits ; the object was to cause a leucocytosis, and increase the bactericidal power of the blood.

For the purpose of these experiments 9 normal rabbits were used. Four were controls, and received intravenous injections of virulent streptococci only ; they all died. The other 5 rabbits received similar doses of streptococci intravenously, but were also given subcutaneous injections of lymph-gland ; all these animals recovered.

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THE INFLUENCE OF TEMPERATURE ON THE SURVIVAL OF PURE SUSPENSIONS OF THE ELEMENTARY BODIES OF VACCINIA.

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Received for publication May 18th, 1934.

It is commonly believed that vaccinia virus in the form of glycerinated lymph or crude skin pulp is readily inactivated when kept at a temperature of 37° C. Gildemeister (1927), for example, states that exposure to this temperature for a few days is sufficient to kill the virus. When highly potent preparations are employed, however, the survival period is certainly longer than this. Carini (1906) found that glycerinated lymph was still active after 10 days' and occasionally after 13 days' incubation at 37° C. Green (1908) compared the survival of crude glycerinated and non-glycerinated lymph, and found that the latter still retained some activity after 12 and sometimes after 20 days at this temperature, whereas the glycerinated material remained active for 12 days, but not longer. When maintained at 10°–15° C. the virus survived for 25 weeks without glycerol, but seldom longer than 12 weeks in glycerol saline. At 3°–4° C. the survival periods of glycerinated and non-glycerinated materials were approximately the same. Glycerol, therefore, has a destructive effect upon vaccinia, and this effect varies directly with the temperature. Its extensive use is probably largely dependent upon the fact that the infecting agents are very susceptible to conditions in autolysing tissues, and that glycerol acts as a desiccant, thus retarding autolysis.

There are a few records concerning the production of so-called thermostable strains of vaccinia. Armstrong (1929) submitted a neuro-testicular strain of virus to gradually increasing periods of incubation at 37° C., passing the virus in the rabbit testis between each exposure to this temperature. By this method of continuous selection and propagation he was able to produce a strain which after 61 transfers remained potent for 33 days at 37° C. It was also found that this increased resistance to heat was accompanied by an increase in virulence. Silber (1932) employed a special buffer mixture containing saccharose, and claimed that calf lymph virus, when suspended in this solution, remained active for periods as long as 66 days at body temperature. Lastly, Steinhardt and Grund (1915), during the course of their earlier work

on the cultivation of vaccinia, found that the virus sometimes remained active for a number of weeks in a simple broth medium.

In view of the work summarized above, the experience of some investigators who have studied the tissue culture method of propagating vaccinia is somewhat surprising. According to Maitland, Laing and Lyth (1932), a period of a few days at incubator temperature is sufficient completely to inactivate the virus in those flasks in which the tissue cells have failed to survive. The susceptibility of the virus to the conditions present in autolysing tissues must again be advanced to account for these findings.

The recent work of Ledingham (1931, 1932), Eagles and Ledingham (1932), Nauck and Paschen (1932) and others on the elementary bodies of vaccinia has provided ample experimental evidence that these bodies represent the actual infecting agents of this virus. Pure suspensions of these elementary bodies in physiological saline can be prepared, and these are now being used with success in the experimental study of vaccinia. During the course of some investigations regarding the conditions necessary for successful cultivation *in vitro*, in which such suspensions were employed, it was found that the bodies, when suspended in a simple nutrient broth medium, were capable of surviving for relatively long periods at incubator temperature. The practical importance of this finding appears to merit the placing on record of some illustrative experiments.

EXPERIMENTAL DATA.

The method of preparing pure suspensions of vaccinia elementary bodies was first described by Eagles and Ledingham (1932). Very briefly it is as follows : A highly virulent strain of dermal lapine is obtained by frequently repeated animal passage. The pulp, removed on the fourth day or even earlier, is very thoroughly ground in a mortar without the addition of sand. A 1 in 40 suspension in distilled water is then prepared, and this is then centrifuged at about 3000 r.p.m. for 15 minutes in order to throw down most of the tissue *débris*. The supernatant fluid is passed through a new Berkefeld V filter, and the filtrate is centrifuged on a high-speed centrifuge at 10,000–12,000 r.p.m. for 30 minutes. Most of the elementary bodies are thereby thrown down as a fine deposit, which is washed by resuspending in distilled water and centrifuging as before. Finally it is suspended in normal saline solution and, after lightly centrifuging in order to deposit any clumps of elementary bodies, the suspension is ready for use. About 5·0 c.c. of suspension can be obtained from 0·5 gm. of rabbit pulp. The infectivity titre of such suspensions is usually very high ; in one instance the titre was 1 in 10,000 million when titrated intradermally on the rabbit in the usual manner.

In order to test the heat resistance of these elementary body suspensions, a 1 in 50 dilution of the original suspension in broth or normal saline solution was prepared. Quantities of 10 c.c. of these broth or saline suspensions in test-tubes plugged with cotton-wool covered by a piece of oiled silk were then placed in the incubator at 37° C. Similar tubes containing the same dilution of the original suspension in broth or saline were stored in the cold room at

0°–4° C. In some experiments samples of the same broth or saline suspension were kept in the dark at room temperature, 12°–17° C. At intervals a small volume was removed from each tube, and the infectivity titre was determined by intradermal titration on the rabbit.

So far, five different elementary body suspensions prepared from the same strain of dermal lapine have been tested, and one suspension prepared from a rabbit testis infected with a neuro-testicular strain of vaccinia. Summaries

TABLE I.

Infectivity Titre of Elementary Body Suspension No. 95 *in Nutrient Broth.*

Duration of storage.	At 37° C.	At 0° C.
Fresh	$0{\cdot}2 \times 10^{-6}$	$0{\cdot}2 \times 10^{-6}$
7 days	10^{-6}	..
16 ,,	10^{-2}	..
36 ,,	10^{-1}	..
46 ,,	Undiluted	..
49 ,,	Ditto*	..

* Confirmed by neutralization test.

TABLE II.

Infectivity Titre of Elementary Body Suspension No. 108 *in Nutrient Broth and in Saline.*

Duration of storage.	In broth at 37° C.	In saline at 37° C.	In saline at 0° C.
Fresh	$0{\cdot}5 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$
8 days	10^{-3}	10^{-1}	10^{-4}
22 ,,	10^{-2}	..	10^{-3}
29 ,,	10^{-1}	..	10^{-3}
37 ,,	..	..	10^{-3}

TABLE III.

Infectivity Titre of Elementary Body Suspension No. 116 *in Nutrient Broth and in Saline.*

Duration of storage.	In broth at 37° C.	In broth at room temperature.	In saline at 37° C.	In saline at 0° C.
Fresh	$0{\cdot}5 \times 10^{-8}$	$0{\cdot}5 \times 10^{-8}$	$0{\cdot}5 \times 10^{-8}$	$0{\cdot}5 \times 10^{-8}$
7 days	$> 10^{-5}$	..	10^{-2}	..
14 ,,	10^{-5}	..	10^{-1}	10^{-7}
21 ,,	10^{-5}	..	*Nil*	10^{-7}
28 ,,	10^{-4}	10^{-5}	..	10^{-7}
34 ,,	10^{-2}	..	..	10^{-6}
41 ,,	10^{-1}*	..	..	10^{-6}
70 ,,	..	10^{-5}	..	10^{-6}
104 ,,	..	10^{-3}	..	10^{-5}

* Confirmation by neutralization test.

Table IV.

Infectivity Titre of Elementary Body Suspension No. 127 *in Nutrient Broth.*

Duration of storage.	In broth at 37° C.	In broth at 0° C.
Fresh	10^{-9}	10^{-9}
3 days	10^{-8}	10^{-8}
7 ,,	10^{-8}	10^{-8}
11 ,,	10^{-5}	10^{-8}
17 ,,	10^{-2}	10^{-8}
25 ,,	10^{-2}	10^{-8}
30 ,,	10^{-1}	..
40 ,,	Undiluted	10^{-6}

Table V.

Infectivity Titre of Elementary Body Suspension No. 146 *(Neurotesticular Strain) in Nutrient Broth.*

Duration of storage.	At 37° C.	At 0° C.
Fresh	10^{-5}	10^{-5}
8 days	10^{-3}	10^{-4}
11 ,,	10^{-3}	10^{-4}
20 ,,	10^{-2}	10^{-3}
30 ,,	Undiluted	10^{-3}

Table VI.

Infectivity Titre of Elementary Body Suspension No. 160 *in Nutrient Broth.*

Duration of storage.	At 37° C.	At 0° C.
Fresh	10^{-8}	10^{-8}
3 days	10^{-8}	10^{-8}
6 ,,	$0{\cdot}2 \times 10^{-5}$	..
9 ,,	$0{\cdot}4 \times 10^{-5}$	10^{-7}
20 ,,	10^{-1}	10^{-7}
32 ,,	Undiluted	..

Table VII.

Infectivity Titre of a Berkefeld V Filtrate of Lapine in Nutrient Broth.

Duration of storage.	At 37° C.	At 0° C.
Fresh	10^{-10}	10^{-10}
3 days	10^{-8}	10^{-8}
7 ,,	10^{-7}	10^{-8}
11 ,,	10^{-6}	10^{-8}
17 ,,	10^{-5}	10^{-8}
25 ,,	10^{-5}	10^{-8}
30 ,,	10^{-5}	10^{-8}
40 ,,	Contaminated	..

of the results obtained in these experiments are given in Tables I–VI. In Table VII is shown, for comparison, the survival of virus in a simple Berkefeld V filtrate of dermal lapine in nutrient broth.

The character of the lesions produced by intradermal inoculation of these incubated suspensions differed in no way from those produced by crude suspensions of lapine. In some experiments a buffered phosphate broth of pH 7·2 or 10 per cent. serum broth were employed instead of simple nutrient broth. These modifications, however, produced no better results than those obtained with the simple medium.

In order to confirm the fact that the intradermal reactions obtained with these suspensions over long periods of incubation were due to the elementary bodies contained in them, a number of serological tests were carried out. In one such experiment the broth with its contained virus, which had been maintained at 37° C. for 50 days, was inoculated intradermally into a rabbit. The skin-lesions were excised aseptically 4 days later, and were ground up with physiological saline. The resulting suspension was then inoculated intradermally into a second rabbit. This animal subsequently developed lesions which were characteristic of vaccinia, and its serum, tested on the tenth day after inoculation, gave a positive vaccinia agglutination reaction to a titre of 1 in 256. Neutralization tests with this recovered virus and a known anti-vaccinial serum were also carried out and the results of these were equally conclusive.

DISCUSSION.

These experiments demonstrate that pure vaccinia virus suspended in a simple broth medium can withstand a temperature of 37° C. for several weeks, while at room temperature the potency remains at a high level for a considerable period. It will be noticed that no attempt was made to obtain by selection a heat-resistant strain of virus. It seems more likely that the capacity to withstand heat depends entirely upon the initial virulence of the strain employed. Whether individual elementary bodies may increase their virulence or whether it is only a question of the number of elementary bodies present it is as yet impossible to say.

The possibility of employing pure suspensions of vaccinia elementary bodies for Jennerian prophylaxis has been under consideration for some time. These findings regarding the heat resistance of these bodies, and their viability when maintained at 0° C., strengthens the belief that these bacteria-free suspensions may be found of value in vaccination practice.

CONCLUSIONS.

Pure suspensions of the elementary bodies of vaccinia in a simple broth medium retain their activity for considerable periods when kept at room temperature, and for several weeks at 37° C.

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THE ATTEMPTED CULTIVATION OF VACCINIA VIRUS IN CONJUNCTION WITH NON-PATHOGENIC MICRO-ORGANISMS.

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Received for publication May 18th, 1934.

THE comparative ease with which vaccinia virus can be cultivated in the presence of living tissue cells, and the repeatedly unsuccessful attempts to cultivate this virus in simple nutrient media, have led many observers to the conclusion that proliferation can occur only within intact living cells. The usual practice is to use cultures of cells obtained from an animal naturally susceptible to the virus or cultures of embryonic tissue, such as chick embryo. The finding of Maitland and Maitland (1928) that vaccinia can be cultivated in a fluid medium consisting of minced chicken kidney in normal chicken serum and Tyrodes' solution has been confirmed by many workers, and their method has been applied to the cultivation of other viruses. In this medium intact living cells can be found for some days, and on this account Rivers, Haagen and Muckenfuss (1929) regard the Maitland technique as a modification of the more usual methods of tissue culture.

Recently Silber and Wostruchowa (1933) have claimed that vaccinia can be readily cultivated in symbiosis with yeasts and other non-pathogenic micro-organisms in a simple nutrient broth medium. Since the present communication is virtually a repetition of the experiments of these workers, it would seem

desirable to state briefly the conception on which their work was based and the methods employed in carrying it out.

They commence by observing that the small size of the virus particles enables them to be adsorbed readily by such substances as kaolin, and also by various bacterial cells as claimed by Dujarric de la Rivière (1930). They therefore suggest that some viruses may actually multiply within the cells of such micro-organisms, particularly within those which the viruses must frequently encounter under normal conditions. In order to test this hypothesis they added a Berkefeld N or Chamberland L3 filtrate of vaccinia lymph to a simple broth medium which had been seeded some hours previously with a culture of *Torula kephyr*. These cultures were then incubated at 37° C., and serial subcultures were made into fresh broth at intervals of three days. Their usual method of testing for the presence of virus was by corneal inoculation of rabbits and guinea-pigs, but intradermal, subcutaneous and intratesticular inoculations were also occasionally employed. No records of quantitative experiments are given. Cultures of the same strain of torula without added virus were used as controls, and these are stated to have produced no lesions when inoculated into the same animals. The reactions obtained by corneal inoculation of subcultures of these mixed torula and virus cultures were considered to be typical of infection with vaccinia. It is further claimed that Guarnieri bodies and Paschen bodies could be demonstrated in the corneal lesions. Intradermal inoculation of these subcultures produced in 48 hours an infiltrated lesion with a necrotic centre, which later developed into an extensive hæmorrhagic erosion. Subcutaneous inoculation produced an even larger infiltration, which reached its height by the third day. Intratesticular inoculation produced an orchitis which was regarded as typical of a vaccinial infection. Inoculation of the subcultures into the scarified skin failed to produce a vesicular eruption ; if, however, the cultures were first ground with sand in a mortar, so as to disintegrate the yeast-cells, a typical vaccinial eruption, in which Paschen bodies were demonstrable, was obtained. The authors suggested that the atypical reactions might be due to coincident proliferation of the yeast-cells. Histological examination of the lesions, however, did not confirm this, since yeast-cells could rarely be demonstrated.

It is stated that the cell-free supernatant fluid obtained by centrifuging these cultures produced no reaction when inoculated into animals, whereas the deposit of yeast-cells, even after repeated washing, gave rise to lesions similar to those produced by the whole cultures. It was therefore concluded that proliferation of the virus takes place entirely within the yeast-cells, and that the dissociation of virus from cells can only be accomplished with considerable difficulty.

One of the culture-tubes representing the eighteenth subculture of the virus was found to be contaminated with *B. subtilis* and with a staphylococcus. Subcultures of this contaminated culture yielded staphylococci only, the yeast and *B. subtilis* having disappeared. Inoculation of this staphylococcus culture into animals produced a reaction similar to those obtained with the yeast cultures. On this evidence it is claimed that vaccinia can be cultivated *in vitro* in conjunction not only with yeast-cells, but also with staphylococci.

Before this report of Silber and Wostruchowa was published, a number of

somewhat similar experiments had been carried out in this laboratory, although the conception of the nature of virus proliferation upon which the experiments were based was entirely different. This work has been continued and amplified in view of the claims made by these workers, but although modern quantitative methods of estimating virus content by intradermal titration on the rabbit have been employed, the results obtained have been entirely of a negative character. Contrary to the findings of Silber and Wostruchowa, no confirmatory evidence has been obtained, either of the adsorption of vaccinia virus by the yeasts or other organisms employed, or of proliferation of virus under the conditions described by these workers. The questions at issue are of so fundamental a character that it appears justifiable to place on record a short account of these researches.

EXPERIMENTAL DATA.

The source of the virus used for these experiments was a highly virulent strain of dermal lapine. From the pulp which was removed on the third or fourth day pure suspensions of vaccinia elementary bodies in physiological saline were prepared by high-speed centrifugalization of Berkefeld V filtrates according to the method described by Eagles and Ledingham (1932). These suspensions represent the virus in a pure and highly concentrated form, and are therefore eminently suitable for experiments of this nature. In a few instances the crude skin pulp was ground in nutrient broth and Berkefeld V filtrates of this material were used in place of elementary body suspensions.

Experiments on the Adsorption of Virus by Bacteria and Yeast-Cells.

In order to test the adsorptive power of various yeasts and bacteria for virus, pure suspensions of the organism in physiological saline were prepared from cultures on solid media. The density of these suspensions was such that when diluted tenfold they matched Tube No. 6 of the Brown opacity scale. To tubes containing 2·0 c.c. of the bacterial or yeast-cell suspension was added 0·1 c.c. of the virus suspension. These tubes were allowed to stand at room temperature for 2 hours and then in the cold room overnight. On the following morning the tubes were centrifuged at about 3500 r.p.m. for 30 minutes, and the clear supernatant fluid (which usually proved to be sterile on cultivation) was removed from the deposit of bacteria or yeast-cells. This deposit was washed by repeated centrifugalization, and was finally resuspended in fresh broth or saline to bring it to the original volume. The supernatant fluid and the washed resuspended deposit were then titrated intradermally on the same rabbit. Controls consisting of the same dilution of virus without added bacteria or yeast-cells and of suspensions of the micro-organism without added virus were inoculated at the same time. It was found that most of the organisms tested produced a reaction when inoculated intradermally into the rabbit, but these could be differentiated from a true vaccinial reaction by their earlier appearance and by the type of reaction produced. The results of three representative experiments are given in Tables I to III:

Table I.

Virus: Vaccinia elementary body suspension.
Adsorbent: *Staphylococcus albus* isolated from lapine.

Degree of Intradermal Reaction on the Fifth Day after Inoculation.

Dilution.	Supernatant after adsorption.	Control: No added bacteria.
10^{-1}	++	++
10^{-2}	++	++
10^{-3}	++	+
10^{-4}	+	+
10^{-5}	+	±
10^{-6}	±	±

The supernatant fluid proved to be bacteria-free.

Table II.

Virus: Vaccinia elementary body suspension.
Adsorbent: *Torula kephyr* (Baarn strain).

Degree of Intradermal Reaction on the Fifth Day after Inoculation.

Dilution.	Supernatant after adsorption.	Control: No added torula cells.
10^{-1}	++	++
10^{-2}	+	+
10^{-3}	±	±
10^{-4}	±	±

The supernatant fluid yielded a few torula colonies when plated on glucose agar.

Table III.

Virus: Berkefeld V filtrate of lapine in nutrient broth.
Adsorbent: *Torula kephyr* (Silber).

Degree of Intradermal Reaction on the Fifth Day after Inoculation.

Dilution.	Virus + torula supernatant.	Virus + saline supernatant.	Virus + torula deposit.	Torula + saline deposit.
10^{-1}	++++	++++	++	++
10^{-2}	+++	+++	+	+
10^{-3}	++	++	+	±
10^{-4}	+	+	−	−

The supernatant fluid proved to be free of torula.

All the experiments of this type yielded the same result. The supernatant fluid obtained by centrifuging the mixture of adsorbing cells and virus contained as much virus as the control, while the thrice-washed deposit produced mild

lesions, which differed but slightly from those produced by a suspension of the organism used as adsorbent without added virus.

It should be noted that the yeast-cell or bacterial suspensions employed in these experiments were considerably denser than three-day cultures of the same organism in nutrient broth. The failure to obtain evidence of any appreciable adsorption under such favourable conditions is therefore all the more significant.

Cultivation Experiments.

Since it was proposed to estimate quantitatively the virus content of the cultures by intradermal inoculation of rabbits, it was first necessary to know the type and degree of skin reaction produced by the various organisms which were to be used as symbionts. As was expected, most of these, although non-pathogenic in the sense that they produced no signs of a generalized infection, yet gave rise to large, sometimes necrotic lesions when introduced into the skin. Of the many bacteria tested, *C. hofmanni*, *Sarcina lutea* and *Bact. acidi lactici* were alone sufficiently innocuous to serve the required purpose. Many species of true yeasts and torulæ were found to be unsuitable for the same reason. Through the courtesy of Prof. Silber the identical strain of *Torula kephyr* which was used by him in his own researches was available, and this was employed in many of the experiments here recorded. Cultures of this strain of *Torula kephyr* grown in nutrient broth at 37° C. for 72 hours, produced definite lesions when injected intradermally. After 24 hours these consisted of well-defined red, markedly raised, circumscribed lesions which, even at this early stage, often showed a pale necrotic centre. This necrotic process progressed and was well marked after 48 hours. Usually the central necrotic area became a caseous mass, which was slowly absorbed, but on one occasion the overlying epithelium broke down to form an ulcer. In a few instances small slightly raised nodules, apparently metastatic in origin, appeared in the skin at some distance from the site of the primary lesion. A more dilute suspension of the same organism produced lesions which could only be differentiated macroscopically from true vaccinial reactions by their earlier appearance. The histological appearance was, however, readily distinguishable from that of a vaccinial infection.

In sections of the lesions removed 48 hours after inoculation the central area of necrosis was seen to be composed almost entirely of polymorphonuclear cells, which appeared to be surrounding and engulfing the yeast-cells. In the lesions removed at a later period histiocytes had appeared at the perphery, and the polymorphs had degenerated into ill-defined bodies with fragmented and deeply-staining nuclei. In sections stained by the eosin-methylene-blue method the coarsely granular oxyphil material of the polymorph cells was well demonstrated. Masses of this pink-staining material derived from the degenerating polymorphs, and later engulfed by the histiocytes, might possibly have been mistaken for inclusion bodies by an observer not familiar with the appearance of the latter.

The medium used for cultivation was either a simple nutrient broth (pH 7·2) or a buffered phosphate broth (pH 7·2). A volume of 9·8 c.c. of the medium

in a hard glass test-tube was inoculated with the organism to be tested, and the culture was incubated at 37° C. for 2–3 hours. A volume of 0·2 c.c. of a vaccinia elementary body suspension was then added and the tubes were returned to the incubator. The controls consisted of a culture of the same organism without the addition of virus and of the virus alone in broth. At intervals of 3 or 4 days subcultures were prepared by transferring 0·2 c.c. of the well-shaken broth culture to 9·8 c.c. of fresh medium and incubating as before. The same procedure was carried out with the control tubes. The original virus suspension, the primary culture and each subsequent subculture were titrated intradermally in the rabbit. In order that the experimental conditions might correspond to those of Silber and Wostruchowa, the bacteria or yeast-cells were usually not removed by centrifuging prior to titration. In a few preliminary experiments in which this procedure was carried out the results obtained with the cell-free supernatants again demonstrated that the virus had not been adsorbed by the bacteria or yeast-cells. Protocols of four representative experiments are given in Tables IV to VII.

TABLE IV.

Virus: Vaccinia elementary body suspension. Organism: *Torula kephyr* (Silber).
Medium: Nutrient broth pH 7·2.
Dilution of virus at each subculture, 1 in 50.

Infectivity Titre.

	Virus + torula at 37° C.	Virus only at 37° C.
Primary culture before incubation	10^{-8}	10^{-8}
Primary culture after 3 days' incubation	10^{-7}	10^{-8}
First subculture after 3 days' incubation	10^{-4}	10^{-4}
Second subculture after 3 days' incubation	10^{-2}	10^{-2}
Third subculture after 3 days' incubation	*Nil*	*Nil*

TABLE V.

Virus: Vaccinia elementary body suspension.
Organisms: *Torula lactosa*, *Agalactia* sp., and *Pleuropneumonia* sp.
Dilution of virus at each subculture, 1 in 50.

Infectivity Titre.

	Virus + *Torula lactosa* in plain broth.	Virus + *Agalactia* in serum broth.	Virus + *Pleuropneumonia* in serum broth.	Virus only in plain broth.
Primary culture before incubation	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Primary culture after 4 days' incubation	10^{-3}	10^{-3}	10^{-4}	10^{-4}
First subculture after 3 days' incubation	Undiluted	10^{-1}	10^{-1}	10^{-1}
Second subculture after 3 days' incubation	*Nil*	*Nil*	*Nil*	*Nil*

TABLE VI.

Virus: Vaccinia elementary body suspension.
Organisms: *C. hofmanni*, *B. acidi lactici*, *Sarcina lutea*.
Dilution of virus at each subculture, 1 in 50.
Medium: Plain broth (pH 7·2).

Infectivity Titre.

	Virus + *C. hofmanni*.	Virus + *B. acidi lactici*.	Virus + *Sarcina lutea*.	Virus alone in broth (control).
Primary culture before incubation	10^{-6}	10^{-6}	10^{-6}	10^{-6}
Primary culture after 4 days' incubation	10^{-3}	10^{-3}	10^{-5}	10^{-5}
First subculture after 4 days' incubation	Undiluted	Undiluted	10^{-1}	10^{-3}
Second subculture after 4 days' incubation	*Nil*	*Nil*	*Nil*	10^{-1}

TABLE VII.

Virus: Elementary body suspension.
Organism: *Torula kephyr* (Baarn).
Media: Plain broth, serum broth and glucose broth.

Infectivity Titre.

	Virus + torula in serum broth.	Virus + torula in plain broth.	Virus + torula in glucose broth.	Virus in broth alone (control).
Primary culture before incubation	10^{-8}	10^{-8}	10^{-8}	10^{-8}
Primary culture after 4 days' incubation	10^{-6}	10^{-6}	10^{-4}	10^{-7}
First subculture after 4 days' incubation	10^{-4}	10^{-4}	10^{-3}	10^{-5}
Second subculture after 4 days' incubation	10^{-1}	10^{-1}	10^{-1}	10^{-2}

DISCUSSION.

The conception of a virus such as vaccinia, having the ability to parasitize other unicellular organisms, has probably arisen from certain current beliefs concerning the nature and action of bacteriophage. On theoretical grounds the chief objection to this is the fact that most viruses are highly specific in their action, proliferating only within the tissues of a few closely related hosts. It therefore seems improbable that vaccinia, while capable of infecting a variety of animal species, could readily adapt itself to the very different environment of a bacterial or yeast-cell. It must be admitted that certain viruses, such as that of yellow fever, normally require an intermediate host, the tissues of which are entirely different from those of the definitive host; yet in these cases also the specificity for the intermediate host is equally strong.

The quantitative experiments described in this paper have failed to produce any evidence in support of this thesis. It therefore only remains to attempt some explanation of the apparently equally definite positive results obtained by Silber and Wostruchowa. These workers based their conclusions upon the appearance of the lesions produced by their cultures, and upon the finding of Guarnieri bodies and Paschen bodies in those lesions. It may be fairly objected that the hæmorrhagic and necrotic lesions produced by interdermal and subcutaneous inoculation were not typical of an infection with a dermal strain of vaccinia. The supposition that these atypical reactions were produced by the combined effect of virus and yeast is unlikely, since Silber and Wostruchowa themselves state that yeast-cells could very rarely be demonstrated in sections of the infected tissues. With regard to the finding of inclusion bodies and elementary bodies no comment is possible, since the recognition of these depends entirely upon the experience of the observer. The only satisfactory method of proving the presence of virus, would be to demonstrate that specific antivaccinial immune bodies are produced in animals which have been inoculated with remote subcultures, and that the active agent in such cultures can be neutralized with a known antivaccinial serum. Until such complete experimental evidence can be produced, the claims for successful cultivation of vaccinia virus in conjunction with bacteria and yeasts must remain in doubt.

SUMMARY.

1. Vaccinia virus is not adsorbed to any appreciable extent by any of the non-pathogenic bacteria or yeasts which have been tested.

2. No evidence has been obtained to support the claim that vaccinia virus can be readily cultivated *in vitro* in conjunction with bacteria or yeasts.

The author wishes to express his thanks to Prof. L. A. Silber, who kindly supplied a culture of his strain of *Torula kephyr*; and to Dr. R. StJohn-Brooks, who supplied many of the bacterial and yeast cultures used in this investigation.

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